of sorting. A total of 4 rounds of sorting was performed, with the first 2 sorts in enrichment mode (high recovery of all positive clones) and the last 2 sorts in purification mode (coincident negative cells rejected). Immediately following the last sort, the collected cells were re-sorted and plated on selective plates to isolate individual clones.

EXAMPLE 28

Rescue and sequencing of mutant scFv-KJ16 genes

Plasmids from scFv-KJ16 yeast (wt and 2 mutants) were rescued according to the protocol described by Ward (Ward, 1991), except that cells were disrupted with a bead beater (BioSpec Products, Inc., Bartlesville, OK) for 2 minutes instead of vortexing. Cells were centrifuged for 1 minute and the upper (aqueous) phase collected. A Wizard® miniprep kit (Promega, Madison, WI) was used to prepare the plasmid DNA and *E. coli* DH5α competent cells (GibcoBRL, Gaithersburg, MD) were transformed with 1 ml of the DNA preparation using the $CaCl_2$ method. Transformations were plated on LB-AMP50. Sequencing of wt scFv-KJ16 and two mutants (mut4 and mut7) was performed using primers that flank the scFv of the display vector and fluorescence automated sequencing (Genetic Engineering Facility of the UIUC Biotechnology Center).

EXAMPLE 29

TCR Binding by Yeast Cell Surface scFv

The monoclonal anti-TCR antibody KJ16 recognizes a conformational epitope on the Vb8 chain of the TCR (Brodnicki et al., 1996). KJ16 has been used for many *in vivo* studies in mice, including efforts to target and delete the Vb8 population of T cells (Born et al., 1987, McDuffie et al., 1986, Roehm et al., 1985). To evaluate the possible effects of varying antibody affinity in mediating these effects, the use of a yeast display system to identify KJ16 variants with increased affinity for TCR was examined. The scFv gene from the anti-TCR antibody KJ16 has been cloned previously and the scFv protein exhibited approximately the same affinity, $K_D \sim 120$ nM, as KJ16 Fab fragments (Cho et al., 1995).

The scFv-KJ16 coding sequence was subcloned so as to be expressed as a fusion polypeptide with the Aga2p agglutinin subunit expressed on the yeast cell surface. The fusion polypeptide includes a hemagglutinin (HA) epitope tag N terminal to the scFv and a c-myc epitope tag at the carboxy-terminus. The inclusion of these epitopes allows monoclonal anti-HA (12CA5) and anti-c-myc (9E10) antibodies to be used in flow cytometry to quantify surface expression of the full length scFv independently of antigen-binding activity. Such normalization helps account for the effects of cell-to-cell variability in surface levels of the fusion polypeptide. As discussed below, the availability of two independent epitope tags can also control for the selection of individual epitope mutants that might not be desired in screening for ligand binding mutants. To evaluate the binding properties of cell surface scFv, a soluble single-chain Vb8-Va3 TCR (Schodin et al., 1996) was biotinylated and the bound ligand was detected with a phycoerythrin-streptavidin conjugate.

Figure 12 shows that yeast transformed with the scFv-KJ16/Aga2 plasmid expressed the HA epitope (Figure 12A) and the c-myc epitope (Figure 12B). Control yeast transfected with only the Aga2p/HA expression vector were positive for the anti-HA Mab but not for the anti-c-myc antibody. The fraction of cells in the non-fluorescent population has been found to depend on plasmid stability and culture growth phase, but the physiological processes that are involved are unknown. Nevertheless, decreasing the induction temperature to 20°C and decreasing the induction time to less than two culture doublings produces populations with >75% of the cells displaying the scFv-KJ16. scFv-4-4-20 was displayed with this system with approximately the same proportion of positive cells.

Binding of biotinylated scTCR to cell surface scFv was also detected by flow cytometry (Figure 12C). The fraction of cells that expressed active scFv was similar to that detected with anti-HA and c-myc antibodies, consistent with the expression of full length, properly folded scFv. Furthermore, two-color histograms demonstrated a tight correlation of scTCR binding with both HA and c-myc epitope display. Biotinylated-scTCR binding is specific to yeast displaying the scFv-KJ16, and was completely inhibited by excess soluble KJ16 IgG (Figure 12D).

The approximate affinity of the surface displayed scFv-KJ16 was determined *in situ* on the cell wall by titrating whole cells with varying concentrations of biotinylated scTCR. Equilibrium binding was measured by analyzing cell-bound scTCR by flow cytometry. Scatchard analysis of the binding data (Figure 13) yielded a K_D of 500 nM, within five fold of that observed for soluble scFv-KJ16. Such agreement is reasonable, since K_D was calculated under the assumption that 100% of the scTCR was active, likely to be an overestimate (i.e. if only 20% were correctly folded, then the surface scFv would have a $K_D \sim 100$ nM). Previously, a substantial fraction of the scTCR purified from solubilized *E. coli* inclusion bodies is incorrectly folded was found (Schodin et al., 1996).

EXAMPLE 30

Selection of Mutagenized scFv-KJ16/Yeast by Fluorescence-Activated Cell Sorting

An *E. coli* mutator strain has been used to mutagenize an scFv for affinity maturation by phage display (Low et al., 1996). This approach was successful in identifying a mutant of scFv-4-4-20 with higher affinity for fluorescein using yeast display. A strength of this mutagenesis approach is its simplicity, requiring only *E. coli* transformation and cell growth. Furthermore, the *E. coli* mutator strain introduces mutations throughout the expression plasmid, and therefore does not bias changes to portions of the scFv believed to be important for determining binding characteristics. Whether this aspect of mutator strain mutagenesis is advantageous depends on the ability to identify key residues that might influence antigen binding, based on available structural information. Examination of published affinity maturation studies suggest that the location of such residues, generally in non-contact residues, is not yet predictable a priori (Hawkins et al., 1993, Patten et al., 1996, Schier et al., 1996, Thompson et al., 1996, Yang et al., 1995, Yelton et al., 1995).

To apply this strategy to scFv-KJ16, the scFv-KJ16/Aga2 plasmid was propagated in the *E. coli* mutator strain XL1-Red (Stratagene) for six cycles of growth. This procedure was predicted to introduce an average of two to three point mutations in the scFv coding sequence, based on a mutation rate per cycle of 1 in 2000 bps. The resultant plasmid preparation was transformed into yeast yielding a library size of approximately 3×10^5 transformants. In other work, larger libraries (10^7) have

been obtained by further optimization of transformation procedures and by pooling independent transformations. This number does not represent an upper size limit for library construction, as further efforts at optimization and scaleup could be straightforwardly applied.

The mutagenized yeast library was subjected to four successive cycles of sorting and amplification, using a double stain for anti-c-myc antibody binding (FITC) and biotinylated-scTCR binding (PE). Biotinylated TCR was used at a 1:5000 dilution (~10 nM) that yielded just below the detectable threshold of binding by wt scFv-KJ16/yeast (Figure 13). The two channel fluorescence profiles of the mutated scFv-KJ16 sample after one sorting cycle (Figure 14A) and after four sorting cycles (Figure14B) are shown. Cells that exhibited fluorescence above the diagonal window shown in Figure 14 were collected for regrowth. The rationale for this diagonal window was that in any given round the sort criteria were based on antigen binding per displayed polypeptide fusion. For example, selection based only on higher PE fluorescence levels (i.e. scTCR binding) would include not only those mutants with higher affinity scFv, but those that display a higher density of scFv per yeast cell. The latter mutants would in principle be eliminated by including the anti-c-myc antibody as one of the two parameters to normalize for surface expression variability. The first two sorting rounds were performed in enrichment mode, isolating the ~0.5% of the cell population with the highest fluorescence and not setting the sort software to reject coincidences (two cells in the same sorted droplet). The final two sorting rounds were performed for purity, with high coincidence rejection. After the fourth cycle, cells were resorted immediately and plated. Ten colonies (mut1 - 10) were selected for further analysis.

EXAMPLE 31

Characterization of Mutant scFv-Yeast

Each of the 10 selected mutants were labeled with anti-HA antibody, anti-c-myc antibody, and biotinylated-TCR and was analyzed by flow cytometry (Figure 15). As might be expected, one clone (mut6) appeared phenotypically similar to wt scFv-KJ16/yeast. Another clone (mut7) was found to exhibit higher TCR binding levels, a result confirmed by several independent titrations. Finally, a number

of the mutants (mut1-5, 8, 9) consistently showed reduced binding to the anti-c-myc antibody compared to binding of the anti-HA antibody or the biotinylated scTCR. The presence of this class of mutants could be explained by the diagonal sort window specification: as shown in Figure 14, cells can "move" into the sort window either by increasing scTCR (PE) binding at constant c-myc (FITC) signal, or alternatively by decreasing c-myc (FITC) binding at constant scTCR (PE) signal. The selection of these mutants could be easily circumvented by using both epitope tags in the fusion, HA and c-myc. Thus, by alternating labeling of each of these epitope tags in each round of sorting, diminished binding to one of the epitope tags would not be enriched in consecutive sorting rounds as in this case.

Fluorescence histograms of the presumptive c-myc epitope mutant (mut4), the scTCR binding mutant (mut7) and another mutant (mut10) were compared with the wt scFv (Figure 16). Mut4 (Figures 16A and 16B) showed a reduction in anti-c-myc labeling, mut7 showed enhanced scTCR binding (Figures 16C and 16D), and mut10 did not show a shift in either, but the fraction of cells that were positive was higher than with the wt scFv (Figures 16E and 16F). As shown in Figures 16E and 16F, close to 100% of mut10 cells were positive for each of the agents tested. This contrasts with each of the other mutants (e.g. see mut4 and mut7) which resembled the wt scFv-KJ16 yeast in exhibiting two distinct populations of cells, one with reduced levels of cell surface scFv. Enhanced plasmid stability of mut10 and repeated failures to rescue the expression plasmid from mut10 into *E. coli* suggest that chromosomal integration has occurred with this mutant plasmid. Thus, the altered surface expression characteristics of mut10 appear to be a consequence of integration of the expression plasmid.

Binding affinity to scTCR was estimated for the mutants shown in Figure 16 by titration with soluble biotinylated scTCR (Figure 17). Nonlinear curve fitting of this data indicate unaltered K_D for mut4 and mut10, but a threefold increased affinity for mut7. The increase in mean fluorescence of mut10 is due to the absence of a nonfluorescent tail in the distribution rather than increased scTCR binding, as is evident in Figures 16E and 16F.

EXAMPLE 32

Sequences of Mutant scFv

The nucleotide sequences of the wt-scFv-KJ16 cloned into the yeast display plasmid, and mut4 and mut7 following rescue of the plasmids from yeast was determined (Figure 18). The wt scFv-KJ16 contained two silent changes from the originally published scFv sequence (Cho et al., 1995). These may have been introduced by PCR prior to cloning of the scFv into the yeast display plasmid. The mut4 sequence contained one mutation and mut7 contained two mutations. The only mutation in mut4 was present in the c-myc epitope (Lys to Glu), consistent with its reduced binding by anti-c-myc antibody as described above. Mut7 contained a change from Arg to Lys in a framework region of the V_L region and a change from Ser to Arg in CDR1 of the V_L chain. The latter mutation is consistent with the higher binding affinity observed for mut7.

Phage display has been used for the selection of scFv with higher antigen binding affinity, as well as isolation of new scFv's from naive libraries (Hoogenboom, 1997). However, there have been difficulties in the expression of some mammalian proteins in *E. coli*, in part because of toxicity, codon bias, or folding problems (e.g. Knappik & Pluckthun, 1995, Ulrich et al., 1995, Walker & Gilbert, 1994). Yeast expression can potentially obviate some of these problems, by offering the advantage that proteins can be expressed with eucaryotic post-translational modifications (e.g., glycosylation and efficient disulfide isomerization). Furthermore, phage display does not generally possess the quantitative precision to discriminate between mutants with binding affinity differing by less than five-fold (Kretzschmar et al., 1995). By contrast, fluorescence labeling and sorting allowed the isolation of 4-4-20 scFv clones with only 3 fold increased affinity. Since most large changes in antigen binding affinity result from directed combination of point mutations, each with smaller effects (Hawkins et al., 1993, Schier et al., 1996, Yang et al., 1995), the capability to identify subtle improvements in affinity could be of significant value. With these advantages in mind, the use of a yeast display system for the affinity maturation of an anti-T cell receptor scFv was developed.

A scFv that is specific for the Vb8 region of a mouse TCR was used in order to generate anti-TCR reagents that may ultimately have enhanced T cell targeting

properties *in vivo* (Cho et al., 1997, Cho et al., 1995). The active scFv was expressed as an Aga2p fusion protein on the surface of yeast, with an affinity that was similar to the native scFv (~500 nM compared to 120 nM for the scFv). To select higher affinity scFv, random mutagenesis with a DNA-repair deficient strain of *E. coli* yielded a mutation frequency of ~2 to 3 per 1000 base pairs after six growth cycles. Flow cytometry with fluorescently labeled scTCR and anti-c-myc antibodies was used to sort cells displaying scFv's with increased scTCR affinity. The anti-c-myc antibody was included as a second criteria for selection to control for mutants with increased TCR binding due not to higher affinity but because of higher cell surface expression of the scFv-c-myc fusion. After multiple rounds of selection, three mutant phenotypic classes were observed: 1) reduced binding to the c-myc antibody but unaltered scTCR binding (mut1-5, 8, 9); 2) enhanced binding to the scTCR with unaltered c-myc labeling (mut7); and 3) higher efficiency surface expression due to chromosomal vector integration (mut10).

The isolation of classes of mutants that are represented by mut4 and mut7 could be predicted from the selection criteria illustrated in Figure 14. That is, any mutant cell that was identified above the diagonal sort window boundary could be accounted for by either of the properties described for mut4 and mut7, since either an increase in scTCR (PE) signal or a decrease in c-myc (FITC) signal places a cell in the sorting window. This does not represent a substantial problem for this approach, however, because of the availability of two independent epitope tags. By utilizing the HA and c-myc tags in alternating sorting cycles, progressive enrichment for diminished labeling of one of the epitope tags should not occur.

The isolation of epitope tag mutants highlights an additional application for yeast surface display: mapping of epitopes recognized by monoclonal antibodies. Although alternative strategies that use peptide libraries have been successful in this regard for linear epitopes, the approach described here can be extended to conformational epitopes. Accordingly, a properly folded protein can be displayed on the yeast cell surface and straightforward random mutagenesis as described herein can be applied to identify epitope residues from non-contiguous polypeptide sequence. Since nonfolded proteins are retained and degraded by the eucaryotic secretory quality control apparatus and varied expression levels are

identified by HA or c-myc labeling, false identification of epitope residues should be minimized by this procedure. The described approach is substantially easier than alanine scanning mutagenesis.

It is not clear why mut10 was enriched in this screen, since its average single chain T cell receptor labeling per c-myc labeling was unaltered. It is possible that the higher fraction of positively labeled cells biased this clone for enrichment due to random spillover into the sort window. In any case, neither scTCR or c-myc labeling were different for this clone, and structural rearrangements of the expression plasmid indicate that it had integrated into a chromosome.

The identification of a single unique CDR mutation in mut7 is consistent with the finding that this mutant scFv has enhanced binding to the T cell receptor. Future efforts to obtain only scFv with higher affinity for the T cell receptor (and not c-myc mutants) involves alternate selection with anti-HA and anti-c-myc antibodies to control for cell surface levels of the scFv. This strategy, combined with DNA shuffling techniques among selected mutants (Stemmer, 1994), should allow the isolation of scFv-KJ16 with considerably higher affinity than the wt scFv ($K_D \sim 120$ nM). Such mutant KJ16 scFv's can be used to test T cell signaling kinetic phenomena, as well as targeting of T cell-mediated killing via bispecific antibodies (Cho et al., 1997).

The present invention demonstrates the purposeful isolation of affinity matured antibodies via cell surface display. As described above, off-rate selection was employed to identify mutants with decreased dissociation rates, whereas in the expression of scFv-KJ16, equilibrated antigen binding was used. These two approaches are complementary, and depend on the affinity of the starting scFv. For $K_D > 1$ nM, it is reasonable to pursue the strategy of equilibration with soluble labeled antigen as dissociation rates would be too rapid to allow effective discrimination of kinetic variation. Furthermore, at these lower affinities bulk soluble antigen is not substantially depleted from the labeling reaction mix, given that displayed scFv is present at effective concentrations of approximately 1-10 nM. By contrast, tightly binding antibodies such as 4-4-20 (K_D =0.4 nM) would deplete soluble labeled antigens at concentrations below K_D unless inconveniently large labeling volumes were employed. However, dissociation kinetics for such tightly binding antibodies are slow

enough to enable quenching, sorting, and analysis via manual mixing procedures. Thus, one could employ a strategy whereby scFv's would be affinity matured via cycles of equilibrium-based screening and mutagenesis to reach K_D ~1 nM, followed by cycles of off-rate screening and mutagenesis to obtain still further improvement.

Cell surface display and flow cytometric screening allows selection of clones from a library based on kinetic binding parameters such as K_D and the dissociation rate constant (k_{diss}). Binding parameters of selected mutants may then be quantitatively estimated in situ in the display format without a need for subcloning or soluble expression, as shown in Figure 17. By contrast, selection of phage displayed antibodies often involves increasingly stringent wash and elution conditions, even to the extent of pH 2 and 8 M GuHCl. Such stringency selection has poor quantitative precision and may not always relate directly to binding parameters such as K_D or k_{diss} under ambient or physiological conditions.

Bacterial cell surface display systems have been described (Gunneriusson et al., 1996) for engineering of antibodies and other proteins. These systems possess some of the advantages of the present yeast display system, although they do not provide the post-translational processing capabilities of the eucaryotic secretory pathway. Access of macromolecules to the displayed protein on bacteria may also be restricted by the diffusion barrier presented by the lipopolysaccharide layer (Roberts, 1996). For this reason, binding to soluble protein antigens or epitope tag labeling with monoclonal antibodies is not possible. Surface display systems in cultured mammalian cells are also available but construction and screening of combinatorial libraries for these systems are not as rapid or as versatile as for yeast.

A fairly small library (3×10^5) was screened to isolate the mutants described herein. This does not represent an upper limit on yeast library size. Yeast libraries with 10^7 clones have been constructed and further increases in library size, if necessary, would be attainable. The present invention shows that yeast surface display can be used to isolate a mutant scFv with increased affinity and that mutants with altered mAb epitopes can be enriched or excluded as desired. Further, the K_D can be estimated in situ in the display format without necessitating subcloning and soluble expression. Quantitative optimization of the screening conditions will enable further

improvements in this method. Applications of yeast surface display extend beyond antibody affinity maturation, to the isolation of binding domains from cDNA expression libraries, or isolation of mutant receptors or ligands on the direct basis of kinetic and equilibrium binding parameters.

EXAMPLE 33

Displayability and expression of the T cell receptor in the yeast display system

The present invention is also directed to a new process for engineering the T cell receptor for improved binding properties, e.g., to peptide-MHC complexes or superantigens. This invention establishes a method for displaying a T cell receptor in a yeast surface display library format. This method can be used: 1) in general, to express polypeptides that are not normally expressed on the surface or yeast, and 2) more specifically, to engineer higher affinity T cell receptors for a ligand of choice.

Protein engineering has not reached a level of development that allows rational and directed engineering of increased affinity binding. As a result, approaches have been developed that identify improved mutants from large mutant populations. The most widely used approach is "phage display", which has used to engineer antibodies, especially in the form of linked, "single-chain" antibodies. However phage display methodology has been unable to display single-chain T cell receptors (scTCRs) successfully. This is most likely because folding of isolated single-chain T cell receptors is very inefficient in the absence of the other components of the CD3 complex and the protein folding machinery of the eucaryotic endoplasmic reticulum; the bacterial periplasm is unable to effectively fold these fragments.

The establishment of a yeast surface displayed T cell receptor is illustrated in Figures 19 through 21. A key improvement has been to isolate a mutant T cell receptor which can be displayed in this system. The wild-type T cell receptor is not functionally displayed, as shown by the absence of binding by an antibody (1B2) that is specific for the native conformation of the T cell receptor (Figure 19). By mutating the T cell receptor and screening a library for 1B2 binding, a mutant single chain T cell receptor displayed in yeast was identified. This establishes a system which can now be used to isolate mutant single chain T cell receptors with improved binding properties.

The present invention provides a yeast cell-surface display system successful in expressing the T cell receptor. Second, expression of the full length T cell receptor could only be achieved after randomly mutagenizing the T cell receptor gene and then selecting by flow cytometry for surface expression. This method exploited an evolutionary approach to "correcting" the expression defect in the T cell receptor.

This same approach could be applied to any polypeptide which in its wild-type form is not displayed efficiently. Selection for "displayability" has been reduced to practice for the T cell receptor, as described in examples 33-37. Once displayable mutant versions of the polypeptide are obtained, these versions can then be subjected to the screening processes for improved binding properties that are described in examples 1-32.

Improved T cell receptor molecules are useful in therapies for cancer, sepsis, and autoimmune diseases such as arthritis, diabetes, or multiple sclerosis. For example, soluble forms of high affinity T cell receptors would act as antagonists of detrimental T-cell mediated autoimmune diseases and thereby provide potential treatments for these diseases. Analogous strategies have been successfully employed with a soluble tumor necrosis factor receptor (TNF-R) and forms of this receptor are in clinical trials for septic shock and rheumatoid arthritis (Moosmayer et al., 1995).

In the methods of the present invention, yeast surface display allows single chain T cell receptors to be engineered to bind with high affinity to MHC-peptide complexes or superantigens. Such molecules would find a variety of medical uses. Examples include, but are not limited to: 1) interfering with inappropriate T cell attacks on healthy tissue in autoimmune diseases such as arthritis, diabetes, and multiple sclerosis; 2) interfering with septic shock due to bacterial superantigen that interact with T cells, leading to massive inflammatory reactions; and 3) destruction of tumor cells that bear T cell receptor ligands (e.g. specific tumor peptide/MHC complexes) by using high affinity T cell receptor together with anti-CD3 bispecific agents to redirect T cells to attack the cancerous cells.

Plasmids and strains

The single-chain TCR gene (V(8.2-linker-V(3.1) gene joined by a modified 205 linker (Cho et al., 1995) was subcloned by PCR into the vector pCR-Script (Stratagene, La Jolla, CA) following the manufacturer's protocol. A 6-His epitope tag was included at the carboxy-terminus of the scTCR for purification purposes. The ~800-bp *Nhe*I/*Xho*I fragment containing the scTCR was excised from pCR-Script and ligated into the yeast surface display vector pCT202 containing a nine-residue epitope tag (HA) and the AGA2 open reading frame downstream of the inducible GAL1 promoter. The resultant construct was transformed by the lithium acetate (LiAc) transformation method of Gietz and Schiestl (Gietz et al., 1995) into the *S. cerevisiae* strain BJ5465 (aura3-52 trp1 leu2D1 his3D200 pep4::HIS2 prbD1.6 can1 GAL; Yeast Genetic Stock Center, Berkeley, CA) containing a chromosomally integrated AGA1 controlled by the GAL1 promoter (strain EBY100).

EXAMPLE 34

Production of an scTCR random mutant library

Approximately 50 ng of pCT202/scTCR were transformed into *E. coli* XL1-Red cells (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Following a 1 hour induction in SOC medium, the recovery was centrifuged at 2000 rpm for 5 min. and resuspended in 500 ml of liquid LB medium containing 100 mg/ml ampicillin plus 50 mg/ml carbenicillin (LB-AMP100-CARB50). The resuspension was added to 15-ml LB-AMP100-CARB50 in a 50-ml Erlenmeyer flask and grown at 37°C with shaking. The culture was replenished with a fresh 15-ml LB-AMP100-CARB50 at mid-log phase (OD_{600} (0.2-0.4), then grown to saturation (OD_{600} ~1.0-1.1; this was considered one "cycle" or round of mutation). A small fraction of this culture (0.75 ml) was added to the next cycle (15-ml LB-AMP100-CARB50). After six cycles of growth, Wizard® miniprep (Promega, Madison, WI) DNA plasmid preparations were performed on the 15-ml culture. Approximately 10 mg of pCT202/scTCR DNA from cycle six were transformed into each of 10 tubes of yeast strain EBY100 using the LiAc method. The 10 reactions were pooled after resuspension in 1-ml ddH_2O/tube, 1/10,000 of the pool plated on selective plates to determine transformation efficiency. The library size was approximately 7×10^6. A 50 ml volume of SD-CAA (glucose 2 wt %, Difco yeast nitrogen base 0.67 wt %,

casamino acids 0.5 wt %) was inoculated with the remainder of the culture, grown overnight at 30°C with shaking, passaged to OD_{600} = 0.05, and grown overnight at 30°C to OD_{600} >1.0. Five milliliters of selective galactose medium SG-CAA (where 2% galactose replaces the glucose in SD-CAA) were then inoculated to OD_{600} = 0.5 and grown overnight at 20°C with shaking for ~20-24 h (1-2 doublings).

EXAMPLE 35

Selection of scTCR mutant library by fluorescence-activated cell sorting

Cells were labeled with 25 mL Mab 1B2 (anti-Vb8.2Va3.1; prepared from ascites fluid and conjugated to biotin) at a concentration of 20 mg/ml. Samples were sorted on a Coulter 753 bench with an event rate of ~4,000 cells/sec (Flow Cytometry Center, UIUC Biotechnology Center). A total of 6 x 10^7 cells were examined during the first sorting round, with ~5% of the population collected. The collected cells were regrown between sorts at 30°C in 4 ml selective glucose medium SD-CAA. After ~18-20 hours, recombinant AGA1 + AGA2-scFv expression was induced at 20°C with shaking in 5 ml SG-CAA. A total of 3 rounds of sorting was performed, with the first sort in enrichment mode (high recovery of all positive clones) and the last 2 sorts in purification mode (coincident negative cells rejected). Immediately following the last sort, the collected cells were re-sorted, collected as two separate populations ("high expression" and "low expression"), and plated on selective plates to isolate individual clones. Twenty clones were examined by flow cytometry.

EXAMPLE 36

Induction and detection of mutant scTCR on the yeast surface

Individual clones from the pCT202/scTCR library sorting were grown overnight at 30°C with shaking in 3 ml SD-CAA followed by induction in SG-CAA as described above. Cultures were harvested after (20-24 hours (1-2 doublings) by centrifugation, washed with PBS (10 mM $NaPO_4$, 150 mM NaCl, pH 7.3) containing 0.1% bovine serum albumin and 0.05% azide, and incubated 45 minutes on ice with 25 (L of 10 mg/ml anti-HA Mab 12CA5 (Boehringer Mannheim, Indianapolis, IN), or biotinylated-1B2 Mab (20 mg/ml) prepared from ascites fluid. Cells were washed

with PBS and incubated 30 minutes on ice with either FITC-labeled $F(ab')_2$ goat anti-mouse IgG (1:50; Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) or a streptavidin-phycoerythrin (SA-PE) conjugate (1:100; PharMingen, San Diego, CA). Labeled yeast cells were analyzed on a Coulter Epics XL flow cytometer. Event rate was ~250 cells/sec. Data for 10,000 events was collected, and the population was gated according to light scatter (size) to prevent analysis of cell clumps. Results from the wild type (wt) TCR and several representative TCR mutants are shown in Figure 19. Double mutants containing the combined mutations from several of these isolates were also constructed and the results of flow cytometry of these are shown in Figure 20.

EXAMPLE 37

Rescue and sequencing of mutant scTCR genes

Plasmids from scTCR yeast (wt and 20 mutants) were rescued according to the protocol described by Ward (Ward, 1991), except that cells were disrupted with a bead beater (BioSpec Products, Inc., Bartlesville, OK) for 2 minutes instead of vortexing. Cells were centrifuged for 1 minute and the upper (aqueous) phase collected. A Wizard® DNA Clean-Up kit (Promega, Madison, WI) was used to prepare the plasmid DNA and *E. coli* DH5α ElectroMAX® competent cells (GibcoBRL, Gaithersburg, MD) were transformed via electroporation with 1 ml of the DNA preparation. Transformations were plated on LB-AMP50. Sequencing of wt scTCR and 20 mutants (mTCR1-mTCR20) was performed using primers that flank the scTCR of the display vector and fluorescence automated sequencing (Genetic Engineering Facility of the UIUC Biotechnology Center). Single mutations were found in the TCR for each of the isolates shown (Figure 21).

EXAMPLE 38

Surface display and soluble expression of single, double and triple TCR mutants

The single-site mutants included the following residues: VaL43P (mTCR7), VaL104P (mTCR16), and VbG17E (mTCR15). Combination of these mutations into double or triple mutants yielded surface display of the scTCR at even higher levels.

To examine the mutant scTCRs selected by the yeast surface display in more detail, the single (mTCR7, mTCR15, mTCR16), double (mTCR7/15, mTCR7/16, mTCR15/16) and triple (mTCR7/15/16) mutants were cloned into a yeast secretion plasmid, with a synthetic pre pro region based on a consensus signal sequence (Clements et al., 1991) under the control of the inducible *GAL 1-10* promoter. The constructs were expressed in yeast and the resultant supernatants were monitored in a quantitative 1B2 binding assay as a measure of properly folded TCR protein. The expression levels of 1B2 active scTCR mutants varied from undetectable levels for the wild type scTCR to the triple mutant, that expressed the highest levels (Figure 22). The order of secretion levels was: triple mutant > double mutants > single mutants > wild type. Selected scTCR double mutants were affinity purified using an anti-TCR antibody column (KJ16-Affigel) and the absolute secretion levels using the low copy expression system were found to be approximately 100 mg/L.

EXAMPLE 39

Comparison of TCR Secretion and Surface Display Levels

In order to directly compare the secreted levels of the scTCR in this system with the levels of TCR expressed in the surface display system, the same mutants were examined as Aga-2 fusions on yeast, using flow cytometry. The scTCR's displayed on the surface of yeast were labeled with the anti-HA antibody followed by a fluorescein-labeled secondary antibody and the biotinylated 1B2, followed by streptavidin-phycoerythrin. The resulting fluorescence intensity of the yeast populations were monitored by flow cytometry and determined as a mean fluorescence units (Figure 23A). The level of fluorescence varied among the single, double and triple mutants, but the relative levels were exactly the same as with the yeast secretion system (Figure 23B)(i.e. triple mutant > double mutants > single mutants > wild type). Thus, the yeast display system was capable of identifying those TCR mutants that would be produced in a secretable expression system at high levels, by simply selecting for those that were expressed at higher levels on the surface of yeast.

EXAMPLE 40

Thermal Stability of Soluble TCRs

In order to explore the protein property that might govern the secretion (and display) levels for the mutant scTCR's, the stability of mutant scTCR were investigated by performing thermal denaturation. Yeast scTCR supernatants were incubated at various temperatures for one hour and the protein activity monitored as a percent of 1B2 activity that remained. Because the wild-type scTCR was not expressed in the yeast system, a wild-type scTCR refolded from E. coli inclusion bodies was used for comparison. This scTCR was used as a thioredoxin fusion protein (TRX-TCR), which is predicted to increase the stability and solubility of a protein. The results showed that the single mutants had higher temperatures of thermal denaturation than the TRX-TCR (Figure 24A). In addition, the double and triple mutants were even more stable, having even higher temeperatures of denaturation than either the wild type TCR or the single mutants (Figure 24A).

The kinetics of thermal denaturation for the mutant scTCR's were determined at 46°C in order to compare the rates of denaturation with each mutant (Figure 24B). Again, yeast scTCR supernatants and their 1B2 binding activity were monitored for TCR activity that remained after incubation for various times. The rate for thermal denaturation was highest for the TRX-TCR and lowest for the triple mutant, with the single and double mutants lying in the intermediate range (Figure 24B). Affinity purified mTCR15/16 had similar thermal denaturation kinetics as that determined for mTCR15/16 measured directly in yeast culture supernatants. This indicates that endogenous yeast proteins present in supernatants did not affect the measured denaturation kinetics.

A direct correlation was observed when the amounts of native TCR remaining after one hour at 48°C were plotted against secretion levels for the mutant scTCRs (need Figure 4 from Shusta et al. = Figure 25). Thus, in the case of the TCR, the intrinsic property of stability was a reliable predictor of secretion efficiency and was directly correlated with yeast cell surface levels of the TCR. Because of this correlation, the display system can be used, by selecting for the higher surface levels of a protein, to isolate mutant proteins that exhibit increased stability.

EXAMPLE 41

Post-translational modification and yeast display

The methods of the present invention have identified variants of the 2C scTCR which are readily expressed in yeast and display significantly enhanced thermal stability. In fact, the single-site mutants identified converted what is normally an unstable scTCR into a scTCR that appears to be as stable as covalently, disulfide "stabilized" or chemically crosslinked single-chain antibody fragments (scFv). This is especially important for the TCR, which has potential as an antagonist in the treatment of autoimmune diseases. As heterologously expressed wild-type TCR has previously been very unstable, pharmacokinetics at physiological temperature will be important.

It has been shown that glycosylation can result in increased secretion and thermal stability of proteins. For example, glucoamylase was less stable as the non-glycosylated form produced in *E. coli* than the glycosylated form produced in the yeast *S. cerevisiae*. Therefore, it was possible that the stabilities of the scTCRs produced in yeast, which have one N-linked glycosylation site in the Vα region, would be greater than that of the non-glycosylated TRX-TCR produced in *E. coli*. However, this is unlikely to be the sole explanation, as the double and triple mutants have increased stability over the single mutants and both of these species are glycosylated. In addition, expression of the wild-type scTCR in yeast was not detected, even though it also would be expected to be glycosylated.

The expression levels of the various mutant scTCRs in the secretion system correlated completely with the level of TCR on the surface in the yeast display system. This relationship may arise from the fact that both systems require the same secretory apparatus. Accordingly, mutations that affect protein folding and stability can affect the same steps of protein transport to the outside (i.e. cell surface or secreted). This is a distinct advantage of expressing eukaryotic proteins in yeast (compared to bacteriophage or bacteria), as there are quality control mechanisms to ensure that only properly folded proteins traverse the entire exocytotic pathway.

Mutant scTCR expression levels also correlated with thermal denaturation rates. This effect is similar to those seen with bovine pancreatic trypsin inhibitor, in which mutations that resulted in increased stabilities also led to increases

in secretion levels in yeast. This correlation supports the theory that a protein which has increased stability in folded form is more likely to be properly packaged and exported from the cell rather than being retained and degraded by the eukaryotic quality control machinery. Of course, many other factors such as forward folding rate, disulfide bond formation, and association with endoplastic reticulum protein folding assistants also play important roles. However, as this invention demonstrates, thermal stability appears to be a good measure of secretion competence for a previously unstable and poorly secreted molecule such as the TCR.

Based on the findings presented herein, one could envision a methodology through which a protein which is poorly expressed and/or unstable can be systematically evolved to express at higher levels and/or as a more stable molecule. The gene encoding the protein can be randomly mutated and the library expressed on the surface of yeast. Once displayed, the population can be screened for clones which exhibit high surface concentrations (high mean fluorescence due to a specific probe). As demonstrated with the scTCR, those variants with improved surface expression have the potential to be more stable molecules which express at high levels. Accordingly, yeast surface display can be a powerful tool for increasing the stability of proteins for crystallization, industrial, and medical applications.

The following references were cited herein:

Bassolino-Klimas, D., et al., Protein Science 1:1465-1476, 1992.

Baumgartner, J-D, et al., Immunobiol. 187:464-477,1993.

Berek, C., et al., ImmunoL Rev. 96:23-41, 1987.

Bird, R.E., et al., Science 242:423-426,1988.

Born, et al., (1987) J. Immunol. 138, 999.

Brodnicki, et al., (1996) Mol. Immunol. 33, 253-263.

Brummell, D.A., et al., Biochemis. 32:1180-1187, 1993.

Cappellaro, C., et al., EMBO Journal 13:4737-4744, 1994.

Chang et al., (1994) PNAS USA 91:11408-11412.

Cho, et al., (1997) Bioconj. Chem. 8, 338-346.

Cho, et al., (1995) J. Biol. Chem. 270, 25819-25826.

Choo, Y., & Klug, A. (1995) Curr. Opin. Biotechnol. 6, 431-436.

Clackson, T., et al., Nature 352:624-628, 1991.

Clements, et al., (1991) Gene 106:267-271.

Daniels, D. A., & Lane, D. P. (1996) Methods 9, 494-507.

Davis, G.T., et al., Biotechnology 9:165-169,1991.

de Nobel, H., et al., Trends in Cell Biol. 4:42-45,1994.

Deng, S., et al., J. Biol. Chem. 269:9533-9538, 1994.

Denzin, L.K., et al., Mol. Immol. 30:1331-1345, 1993.

DiRienzo, J.M., et al., Ann. Rev. Blochem. 47: 481-532, 1978.

Droupadi, P.R., et al., J. Mol. Recog. 5:173-179,1993.

Eilat et al., (1992) PNAS USA 89:6871-6875.

Ellman, et al., (1997) Proc. Natl. Acad. Sci. USA 94, 2779-2782.

Engel et al., (1992) Science 256:1318-1321.

Fischman, A.J., et al., J. Nucl. Med.. 34:2253-2263, 1993.

Foote, J., et al., Nature 352: 530-532, 1991.

Francisco, S.A., et al., PNAS 90:10444-10448,1993.

Garrard, L.J., et al., Gene 128: 103-109, 1993.

Georgiou, et al., (1997) Nat. Biotechnol. 15, 29-34.

Gilli, P., et al., J. Phys. Chem. 98:1515-1518, 1994.

Gilson, M.K., et al., Proteins:Struc., Func., Genet. 3:32-52, 1988.

Goldenberg, D.M. et al., Am. J. Med. 94:297312,1993.

Goldenberg, D.M., et al., Int. J Oncol. 3:5-11, 1993.

Greener, A., et al., Strat. in MoL Biol. 7: 32-34, 1994.

Guddat, L.W., et al., J. Mol. Biol. 236:247-274, 1994.

Gunther, R., et al., J. BioL Chem. 268: 7728-7732, 1993.

Hammond, C., Helenius, A. Curr. Opin. Cell Biol. 5:523-529, 1995.

Hand, P.H., et al., Cancer 73:1105-1113,1994.

Hawkins, R.E., et al., J. MoL BioL 234; 958-964,1993.

Hawkins, R.E., et al., Mol. Riol. 226: 889-896, 1992.

Herron, J.N., et al., Biochemistry 25:4602-4609, 1986.

Hibbits, K.A., et al., Biochemistry 33:3584-3590,1994-

Hilzemann, R. TIPS 9:408-411, 1988.

Holland, J.I. Adaptation in Natural and Artificial Systems , MIT Press, Cambridge, 1992.

Holst, M., et al., Proteins: Struct., Func., & Gen. 18:231-245, 1994.

Horwitz, A.H., et al., PNAS 85: 8678-8682, 1988.

Huse, W.D., et al., Science 246: 1275-1291, 1989.

Huston, J.S., et al., PNAS 85:5879, 1988.

Ishikawa, E., et al., J. Clin. Lab. Anal. 7:376-393, 1993.

Johnsson, N., et al., Cell, in press, 1994.

Johnston, M., et al., Mol. Cell. Biol. 4:1440-1448, 1984.

Kang, A.S., et al., PNAS 88:11120-11123, 1991.

Kappler et al., (1994) PNAS USA 91:8462-8466.

Kelley, R.F., et al., Biochemistry 32;6828-6835, 1993.

Kozack, R.E., et al., Protein Sci. 2:915-926, 1993.

Kranz, D.M., et al., J. Biol. Chem. 257:6987-6995, 1982.

Kricka, L.J. J, Clin. Immunoassay 16:267-271, 1993.

Lipke, P.N., et al., Microbiol, Rev. 56:180-194, 1992.

Low, N. M., et al., J. Mol. Biol. 260:359-368, 1996.

Lu, C.-F., et al., J. Cell Biol. 128:333-340, 1995.

Mallender, W.D., et al., J. BioL Chem. 269: 199-206, 1994.

Marks, J.D., Griffiths, A.D., Malmq ' vist, M., Clackson, T.P., Bye, J.M.,

Ward, A.C. Nucl. Acids. Res. 18:5319, '1991.

Winter, G. "By-Passing Immunization: Building High Affinity Human Antibodies by Chain Shuffling." Biotechnology 10:779-783,1992.

Marks, J.D., et al., J. BioL Chem. 267: 16007-16010, 1992.

McKearn, T.J. Cancer 71:4302-4313, 1993,

Miettinen, M. Ann. Med. 25:221-233, 1993.

Moks, T., et al., Biochemistry 26:5239-5244,1987.

Mukkur, T.K.S. CRC Crit. Rev. Biochem. 16:133-167,1984.

Muller, G.W., et al., J. Med. Chem. 35:740-743, 1992.

Near, R.I., et al., Mol Immunol, 30: 369-377, 1993.

Necker et al., (1991) Eur. J. Immunol. 21:3035-3040.

Nell, L.J., et al., Biopolymers 32i 1 1-21, 1992.

Normington, K., et al., Cell 57:1223-1236,1999.

Novotny et al., (1991) PNAS USA 88:8646-8650.

Omelyanenko, V.G., et al., Biochemistry 32:10423-10429, 1993.

Riechmann, L., et al., Biochemistry 32:8848-8855, 1993,

Riechmann, L., et al., MoL Biol. 224:913-918, 1992.

Roberets, S., et al., Nature 328:731-734,1987.

Roy, A., et al., Mol. Cell. Biol. 11:4196-4206, 1991.

Rumbley, C.A., et al., J. Biol. Chem. 268: 13667-13674,1993.

Schreuder, M. P., et al., Yeast 9:399-409, 1993.

Schreuder, M. P.,et al., Trends Biotechnol. 14:115-120, 1996.

Searle, M.S., et al., Anals de Quimica 89:17-26, 1993.

Serafini, A.N. J. Nucl. Med. 34:533-536, 1993.

Sigurskjold, B.W., et al., Eur. J. Biochem. 197:239-246,1991.

Slanetz et al., (1991) Eur. J. Immunol. 21:179-183.

Soo Hoo et al., (1992) PNAS USA 89:4759-4763.

Stemmer, W.P.C. Pro. Nat. Acad. Sci. U.S.A. 91:10747-10751, 1994 a.

Stemmer, W.P.C. Nature .370:389-391, 1994 b.

Stemmer, W.P.C.et al., BioTechniquesl 4:256-265,1993.

Ward, M., et al., Biotechnology 8:435-440, 1990.

Ward et al. (1991) Scand. J. Immunol. 34:215-220.

Williams, D.H., et al., PNAS 90:1172-1178,1993.

Wood, C.R., et al., Nature 314:446-449, 1985.

Wulfing et al. (1994) J. Mol. Biol. 242:655-669.

Yaimush, M., et al., Crit. Rev. Ther. Drug Carr. Sys. 10:197-252, 1993.

Yarmush, M.L., et al., Biotech. Adv. 10:413-446, 1992.

Zaccolo, M., et al., Int. J. Clin. Lab. Res. 23:192-198, 1993.

Zebedee, S.L., et al., PNAS 89-.3175-3179, 1992.

Gietz, et al., (1995) Yeast 11, 355-360.

Gunneriusson, et al., (1996) J. Bacteriol. 178, 1341-1346.

Hoogenboom, H. R. (1997) Trends Biotechnol. 15, 62-70.

Knappik, A., & Pluckthun, A. (1995) Prot. Eng. 8, 81-89.

Kretzschmar, et al., (1995) Anal. Biochem. 224, 413-419.

Ladner, R. C. (1995) Trends Biotechnol. 13, 426-430.

Lowman, et al., (1991) Biochemistry 30, 10832-10838.

Markland, et al., (1996) Methods Enzymol. 267, 28-51.

Matthews, D. J., & Wells, J. A. (1993) Science 260, 1113-1117.

McDuffie, et al., (1986) Proc. Natl. Acad. Sci. USA 83, 8728.

Patten, et al., (1996) Science 271, 1086-1091.

Petsko, G. (1996) Nature 384 (Supplement), 7-9.

Phizicky, E. M., & Fields, S. (1995) Microbiol. Rev. 59, 94-123.

Rabinowitz, et al., (1996) Proc. Natl. Acad. Sci. 93, 1401-1405.

Roberts, I. S. (1996) Annu. Rev. Microbiol. 50, 285-315.

Rode, et al., (1996) Biotechniques 21, 650.

Roehm, et al., (1985) J. Immunol. 135, 2176.

Schier, et al., (1996) J. Mol. Biol. 263, 551-567.

Schlueter, et al., (1996) J. Mol. Biol. 256, 859-869.

Schodin, et al., (1996) Mol. Immunol. 33, 819-829.

Thompson, et al., (1996) J. Mol. Biol. 256, 77-88.

Ulrich, H. D., Patten, P. A., Yang, P. L., Romesberg, F. E., & Schultz, P. G. (1995) Proc. Natl. Acad. Sci. USA 92, 11907-11911.

Walker, K. W., & Gilbert, H. F. (1994) J. Biol. Chem 269, 28487-28493.

Wang, C. I., Yang, Q., et al. (1996) Methods Enzymol. 267, 52-68.

Yang, et al., (1995) J. Mol. Biol. 254, 392-403.

Yelton, et al., (1995) J. Immunol. 155, 1994-2004.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These references are incorporated by reference to the same extent as if each individual publication was specifically and individually incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A method for selecting proteins with enhanced phenotypic properties relative to those of the wild-type protein, comprising the steps of:

transforming yeast cells with a vector expressing a protein to be tested fused to a yeast cell wall protein, wherein mutagenesis is used to a generate a variegated population of mutants of the protein to be tested;

labeling said yeast cells with a first label, wherein said first label associates with yeast expressing said protein to be tested and does not associate with yeast which do not express said protein to be tested;

isolating said yeast cells with which said first label is associated; and

analyzing and comparing said properties of said mutant protein expressed by yeast with properties of said wild-type protein, wherein yeast cells exhibiting mutant proteins with enhanced properties over the wild-type protein are selected.

2. The method of claim 1, wherein said phenotypic properties are selected from the group consisting of surface expression levels, stability, binding constant, and dissociation constant.

3. The method of claim 1, wherein said protein to be tested is an antibody, Fab, Fv, or scFv antibody fragment.

4. The method of claim 1, wherein said protein to be tested is the ligand binding domain of a cell surface receptor.

5. The method of claim 4, wherein said cell surface receptor is a T cell receptor.

6. The method of claim 1, wherein the protein to be tested is fused by its N terminus to the C terminus of said yeast cell wall protein.

7. The method of claim 1, wherein the yeast cell wall protein is an agglutinin.

8. The method of claim 1, wherein said yeast strain is of a genus selected from the group consisting of *Saccharomyces*, *Pichia*, *Hansenula*, *Schizosaccharomyces*, *Kluyveromyces*, *Yarrowia*, and *Candida*.

9. The method of claim 1, wherein said mutants of the protein to be tested are selected from the group consisting of single mutants and multiple mutants.

10. The method of claim 1, wherein said first label is selected from the group consisting of magnetic particles attached to a ligand for the protein to be tested and fluorescent labels.

11. The method of claim 1, wherein selection of mutated proteins of interest with enhanced phenotypic properties involves iterative cycles of said enrichment and labeling steps.

12. The method of claim 1, further comprising the steps of:

labeling said yeast cells with a second label, wherein said vector used to transform said yeast cells contains means for expressing a polypeptide sequence fused to said protein to be tested to produce a fusion polypeptide and said second label associates with yeast cells expressing said fusion polypeptide and does not associate with yeast cells which do not express said fusion polypeptide;

enriching a transformed yeast population by quantitating said second label, wherein an occurrence of said second label is directly proportional to an abundance of said fusion polypeptides expressed on the cell surface; and

comparing said quantitation of said first label to said quantitation of said second label to determine surface expression levels of said protein to be tested.

13. The method of claim 12, wherein an increase in said surface expression levels of said mutant protein to be tested relative to the level surface expression of the wild-type protein tested can be used to select for desirable phenotypic properties of said mutant protein.

14. The method of claim 13, wherein said phenotypic properties are selected from the group consisting of intracellular expression level, stability, binding constant, dissociation constant, level of secretion, and solubility.

15. The method of claim 12, wherein said polypeptide portion of said fusion polypeptide recognized by said second label is an epitope tag.

16. The method of claim 12, wherein said first label and said second label are fluorescent labels.

17. The method of claim 1, further comprising the steps of:

cloning a gene encoding said selected mutant proteins into a vector adapted for expression in a eukaryote; and

expressing said mutant protein in said eukaryote, wherein said enhanced properties of said mutant protein are confirmed by comparing the properties of said enhanced properties of said mutant protein with the properties of said wild-type protein.

18. The method of claim 17, wherein said eukaryote is selected from the group consisting of mammalian, insect and yeast.

19. The method of claim 1, further comprising the steps of:

cloning a gene encoding said selected mutant proteins into a vector adapted for expression in a prokaryote; and expressing said mutant protein in said prokaryote, wherein said enhanced properties of said mutant protein are confirmed by comparing the properties of said enhanced properties of said mutant protein with the properties of said wild-type protein.

20. A method for selecting proteins for displayability on a yeast cell surface, comprising the step of:

transforming yeast cells with a vector expressing a protein to be tested fused to a yeast cell wall protein, wherein mutagenesis is used to a generate a variegated population of mutants of the protein to be tested;

labeling said yeast cells with a first label, wherein said first label associates with yeast expressing said protein to be tested and does not associate with yeast which do not express said protein to be tested;

isolating said yeast cells with which said first label is associated, by quantitating said first label, wherein a high occurrence of said first label indicates said protein to be tested has desirable display properties and wherein a low occurrence of said first label indicates said protein to be tested does not have desirable display properties.

21. The method of claim 20, wherein said protein to be tested is an antibody, Fab, Fv, or scFv antibody fragment.

22. The method of claim 20, wherein said protein to be tested is the ligand binding domain of a cell surface receptor.

23. The method of claim 22, wherein said cell surface receptor is a T cell receptor.

Yeast display mimics *in vivo* affinity maturation

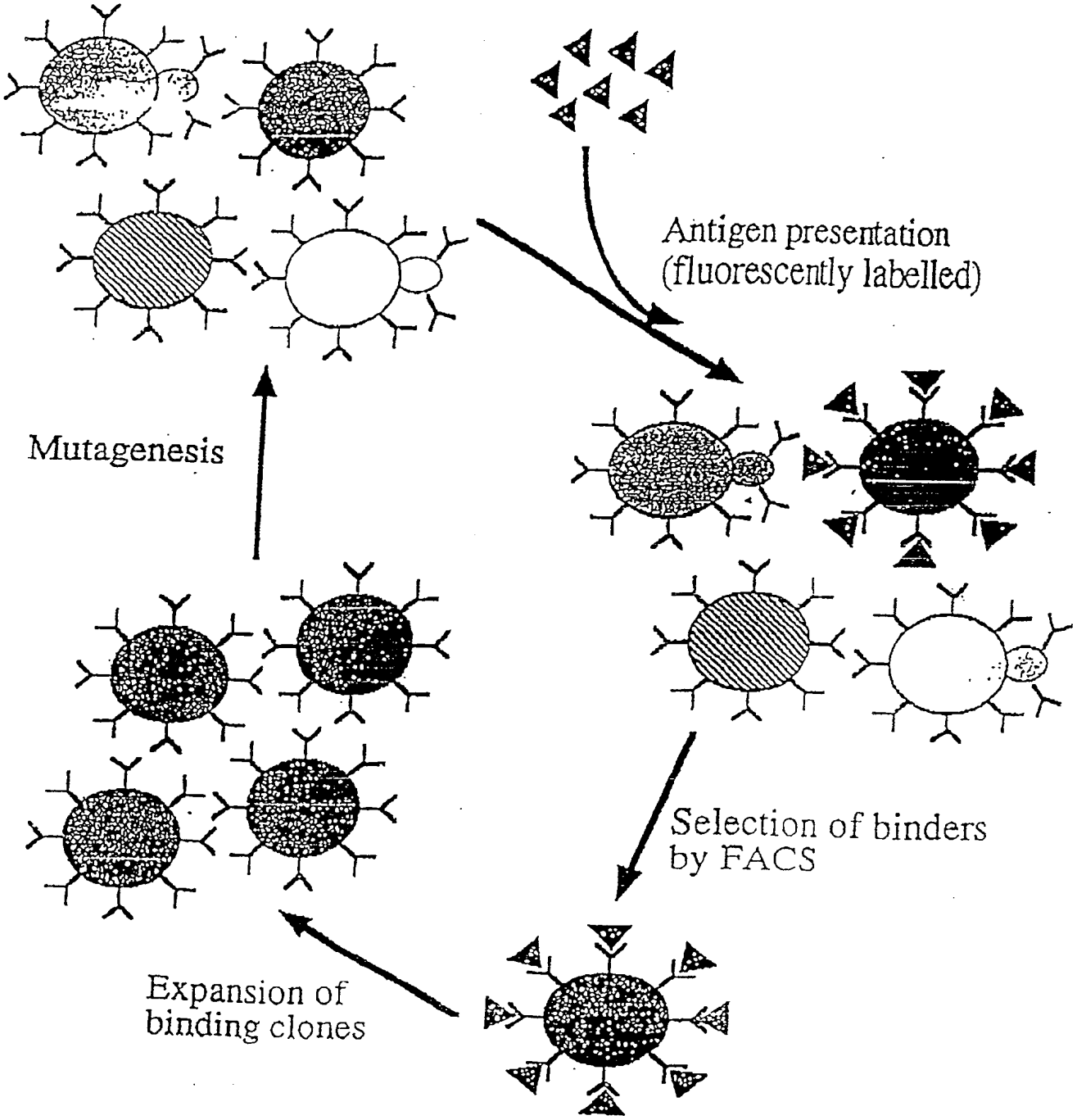


Figure 1

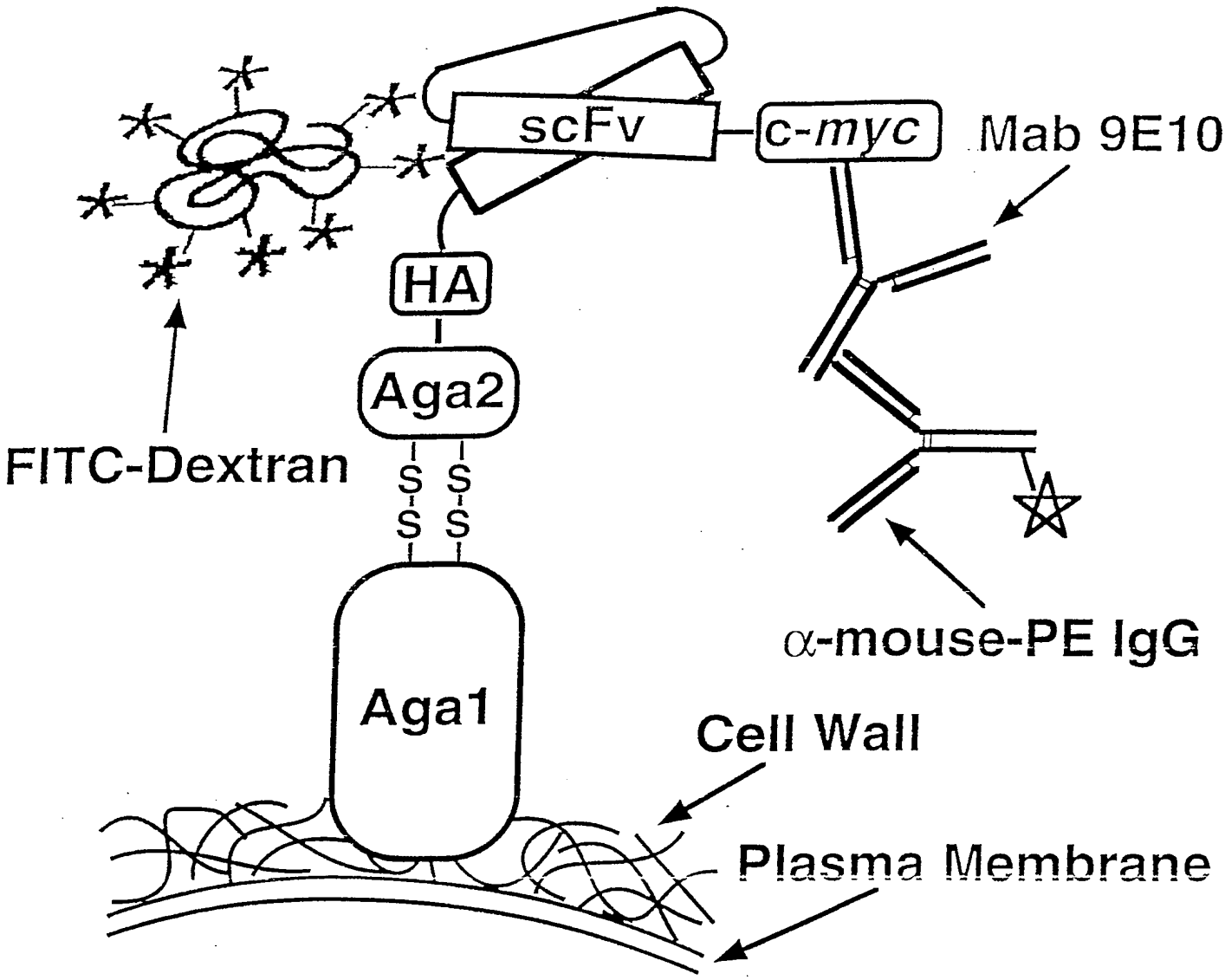
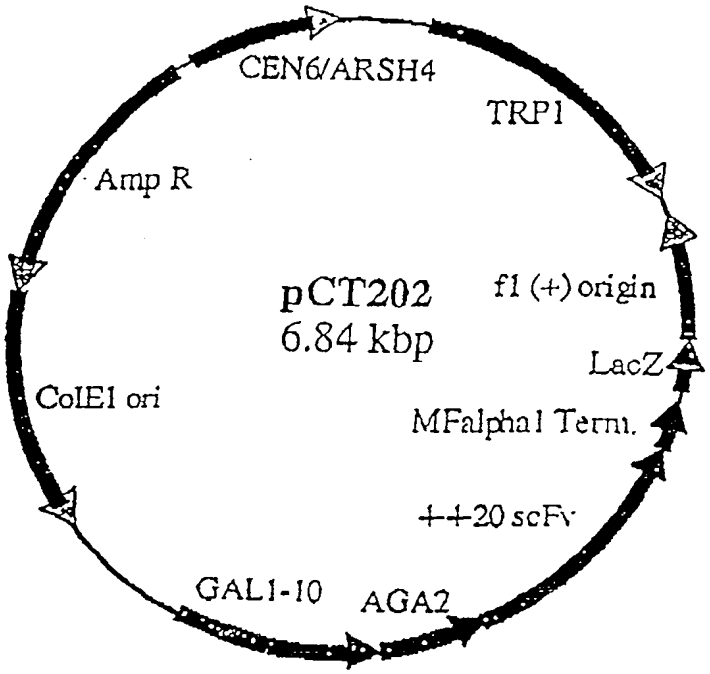
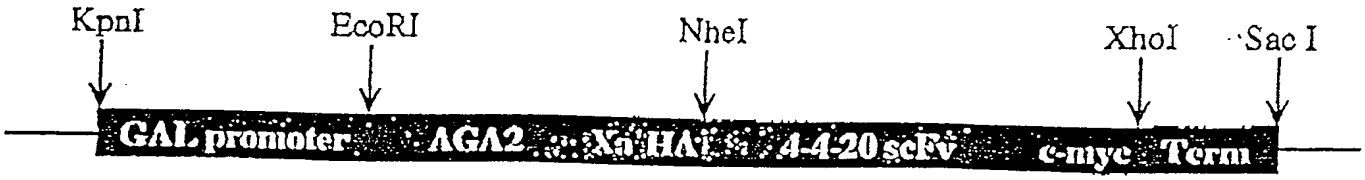

scFv
c-myc
Mab 9E10
HA
Aga2
FITC-Dextran
S S
S S
α-mouse-PE IgG
Aga1
Cell Wall
Plasma Membrane

FIGURE 2

3 Vector for yeast surface display



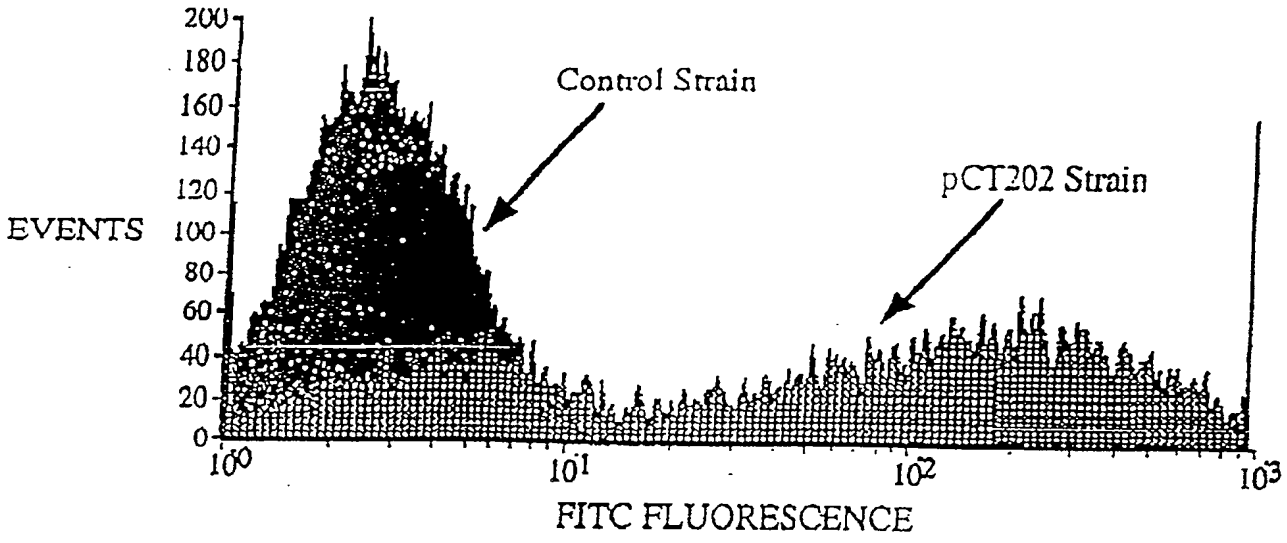


- Transcriptional regulation by galactose
- N-terminal HA and C-terminal c-*myc* epitope tags
- Factor Xa protease cleavage site

Figure 3

4 Displayed fusions can be detected by fluorescence techniques

Flow cytometric histograms of yeast labelled with α-c-myc/α-mouse-PE



Micrographs of yeast labelled with α-c-myc/α-mouse-PE

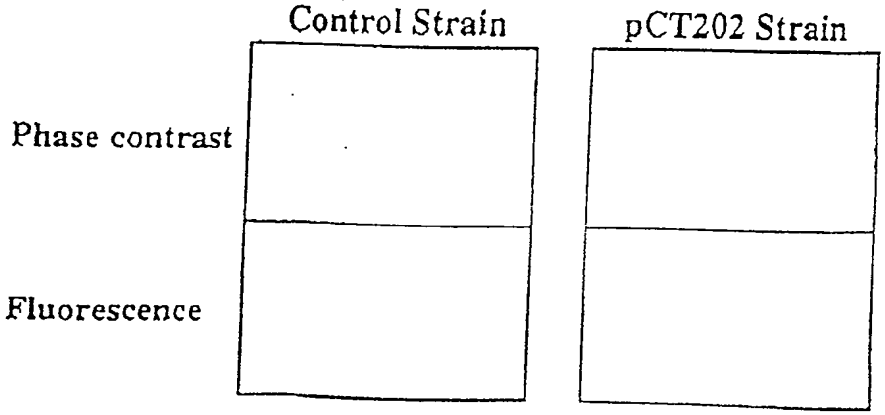
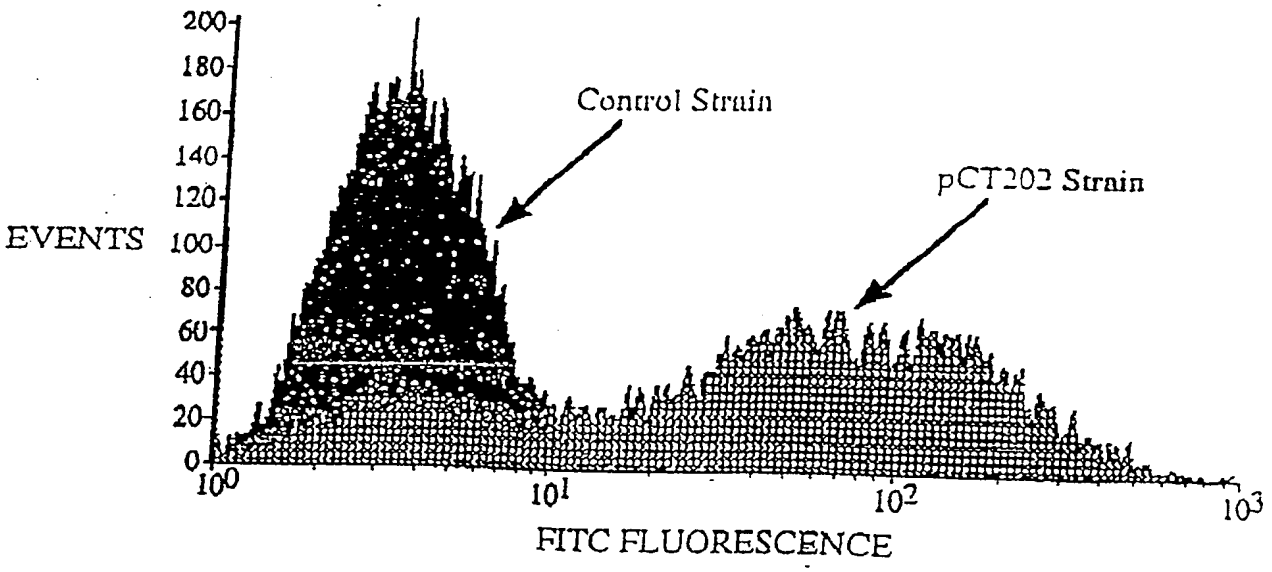


Figure 4

Antigen binding by 4-4-20 scFv can be detected by fluorescence

Flow cytometric histograms of yeast labelled with FITC-dextran ($2x10^6$)



Micrographs of yeast labelled with FITC-dextran (MW $2x10^6$)

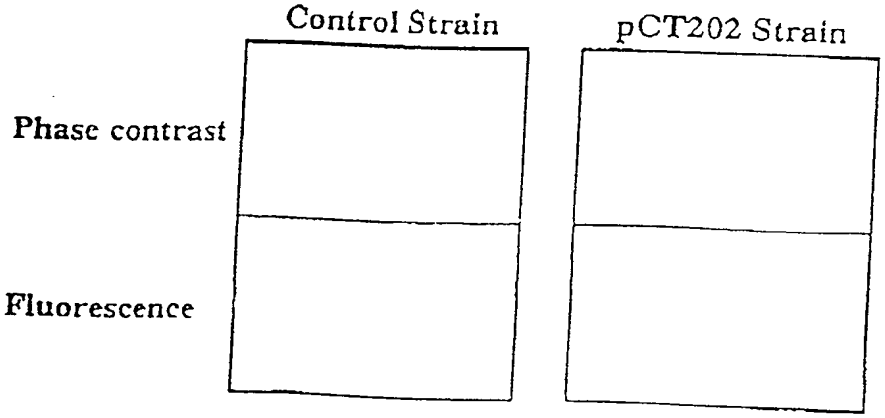
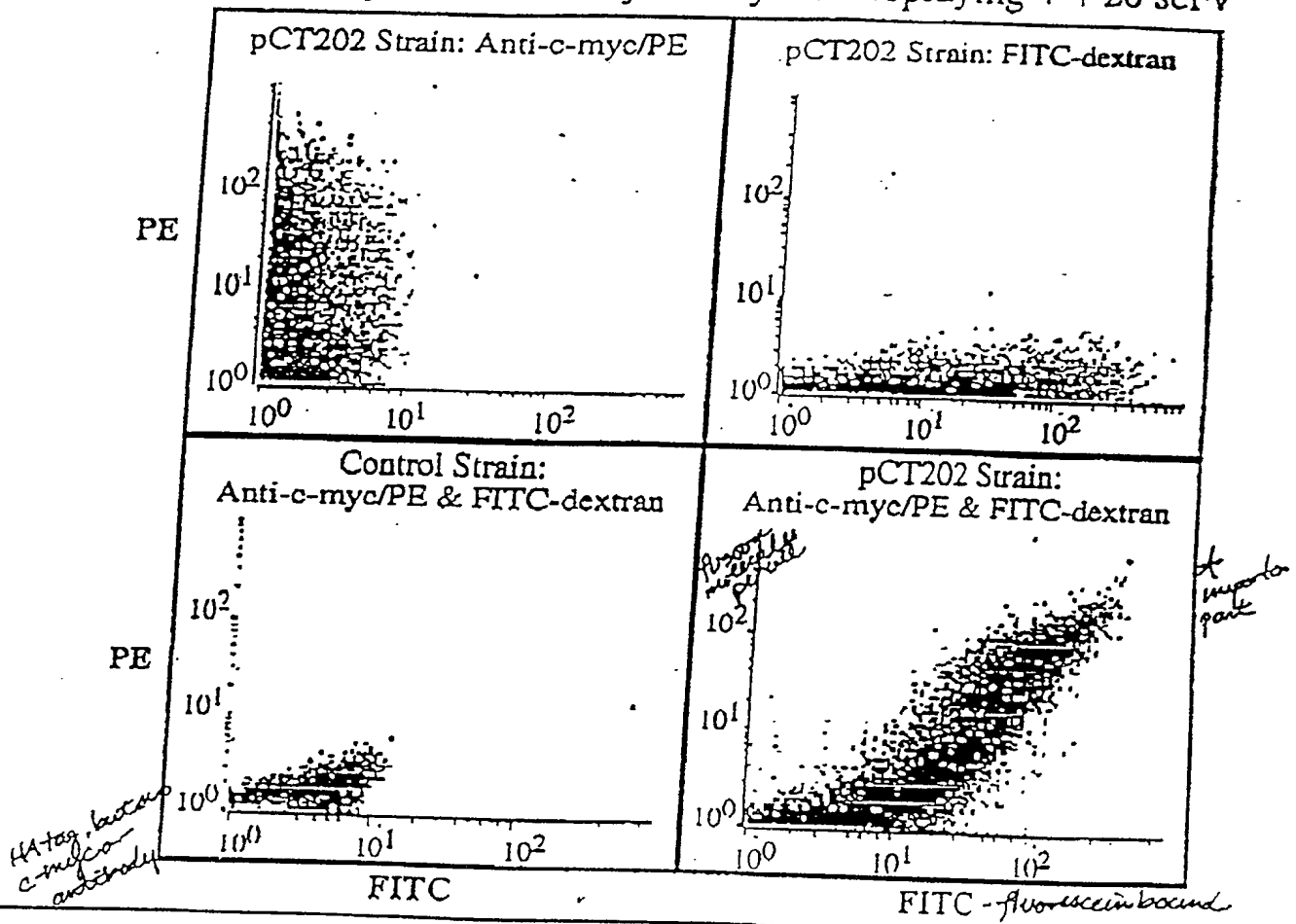


Figure 5

6 4-4-20 activity and c-*myc* can be simultaneously detected

Two-color flow cytometric analysis of yeast displaying 4-4-20 scFv



Micrographs of yeast labelled with anti-c-myc/PE and FITC-dextran

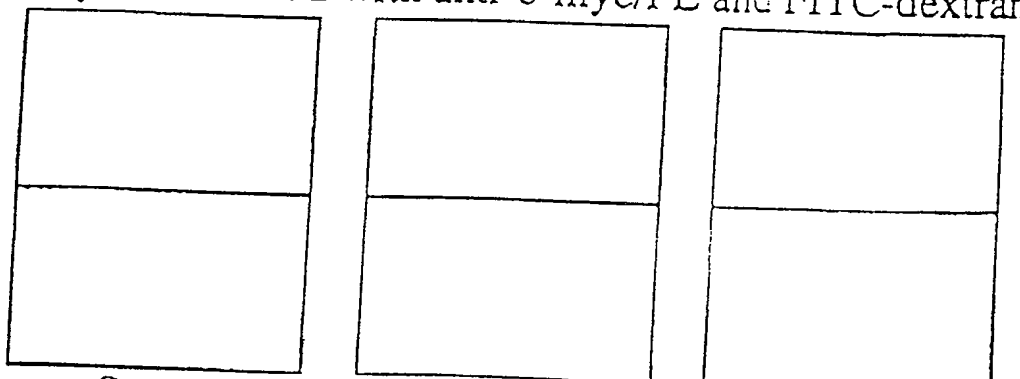


Figure 6

Sequence of AGA2-HA-4-4-20 gene cassette:

```
   1  ATTAGAATTC CCTACTTCAT ACATTTTCAA TTAAGATGCA GTTACTTCGC
  51  TGTTTTTCAA TATTTTCTGT TATTGCTTCA GTTTTAGCAC AGGAACTGAC
 101  AACTATATGC GAGCAAATCC CCTCACCAAC TTTAGAATCG ACGCCGTACT
 151  CTTTGTCAAC GACTACTATT TTGGCCAACG GGAAGGCAAT GCAAGGAGTT
 201  TTTGAATATT ACAAATCAGT AACGTTTGTC AGTAATTGCG GTTCTCACCC
 251  CTCAACAACT AGCAAAGGCA GCCCCATAAA CACACAGTAT GTTTTTAAGG
 301  ACAATAGCTC GACGATTGAA GGTAGATACC CATACGACGT TCCAGACTAC
 351  GCTCTGCAGG CTAGCGACGT CGTTATGACT CAAACACCAC TATCACTTCC
 401  TGTTAGTCTA GGAGATCAAG CCTCCATCTC TTGCAGATCT AGTCAGAGCC
 451  TTGTACACAG TAATGGAAAC ACCTATTTAC GTTGGTACCT GCAGAAGCCA
 501  GGCCAGTCTC CAAAGGTCCT GATCTACAAA GTTTCCAACC GATTTTCTGG
 551  GGTCCCAGAC AGGTTCAGTG GCAGTGGATC AGGGACAGAT TTCACACTCA
 601  AGATCAGCAG AGTGGAGGCT GAGGATCTGG GAGTTTATTT CTGCTCTCAA
 651  AGTACACATG TTCCGTGGAC GTTCGGTGGA GGCACCAAGC TTGAAATTAA
 701  GTCCTCTGCT GATGATGCTA AGAAGGATGC TGCTAAGAAG GATGATGCTA
 751  AGAAAGATGA TGCTAAGAAA GATGGTGACG TCAAACTGGA TGAGACTGGA
 801  GGAGGCTTGG TGCAACCTGG GAGGCCCATG AAACTCTCCT GTGTTGCCTC
 851  TGGATTCACT TTTAGTGACT ACTGGATGAA CTGGGTCCGC CAGTCTCCAG
 901  AGAAAGGACT GGAGTGGGTA GCACAAATTA GAAACAAACC TTATAATTAT
 951  GAAACATATT ATTCAGATTC TGTGAAAGGC AGATTCACCA TGTCAAGAGA
1001  TGATTCCAAA AGTAGTGTCT ACCTGCAAAT GAACAACTTA AGAGTTGAAG
1051  ACATGGGTAT CTATTACTGT ACGGGTTCTT ACTATGGTAT GGACTACTGG
1101  GGTCAAGGAA CCTCAGTCAC CGTCTCCTCA GAACAAAAGC TTATTTCTGA
1151  AGAAGACTTG TAATAGCTCG AG
```

FIGURE 7

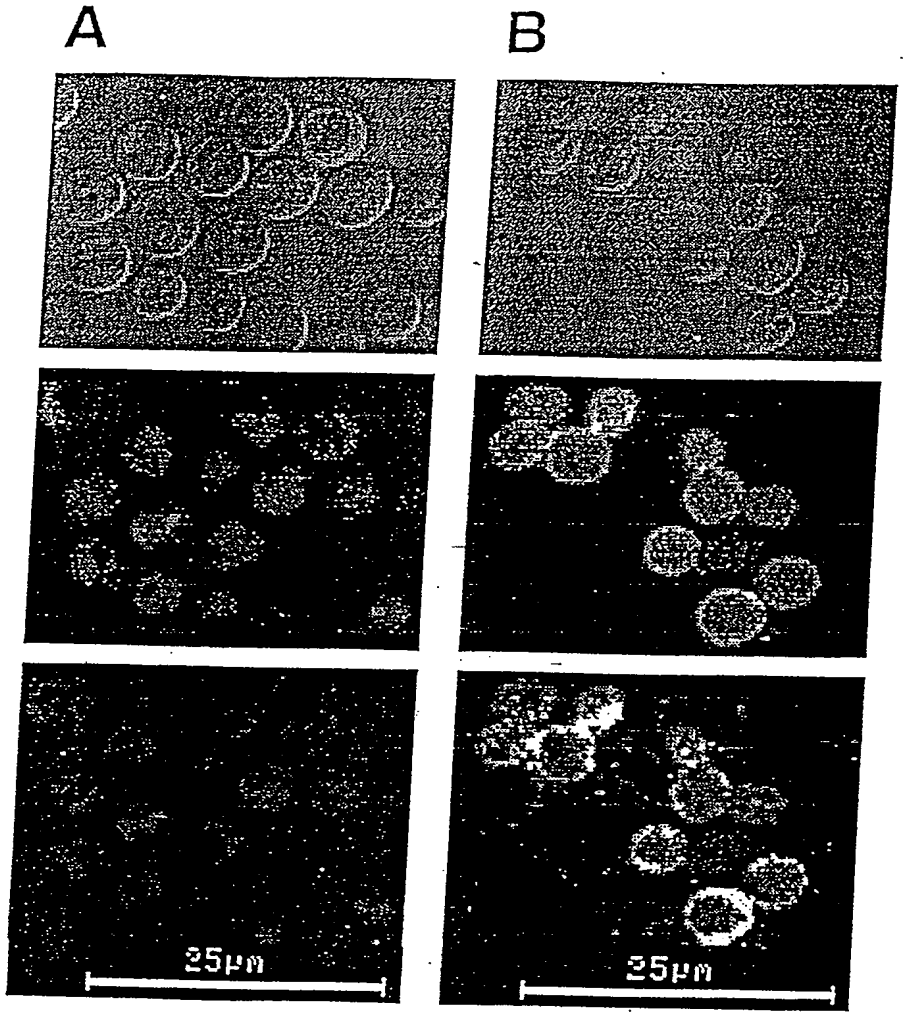


Figure 8

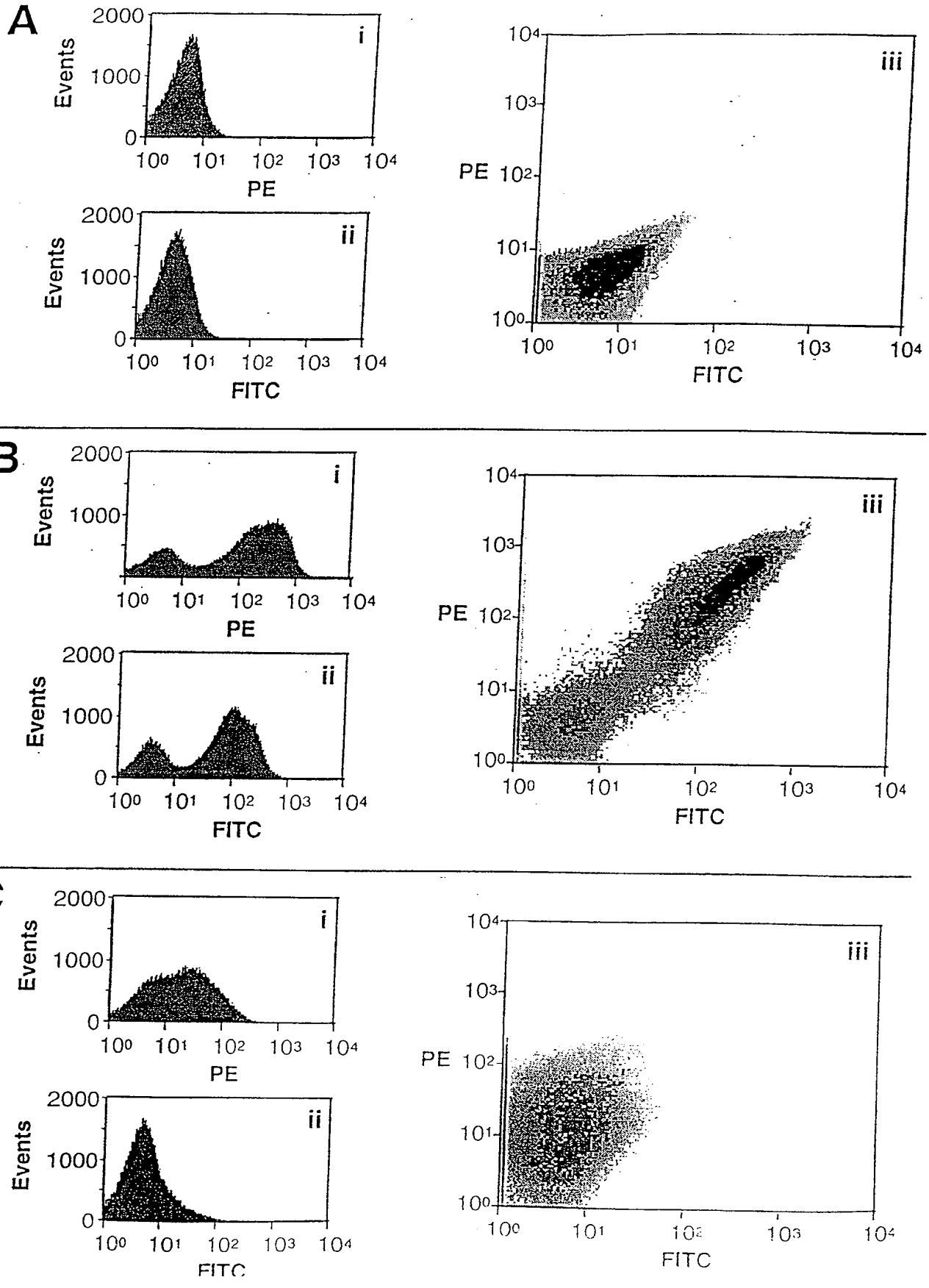


Figure 9

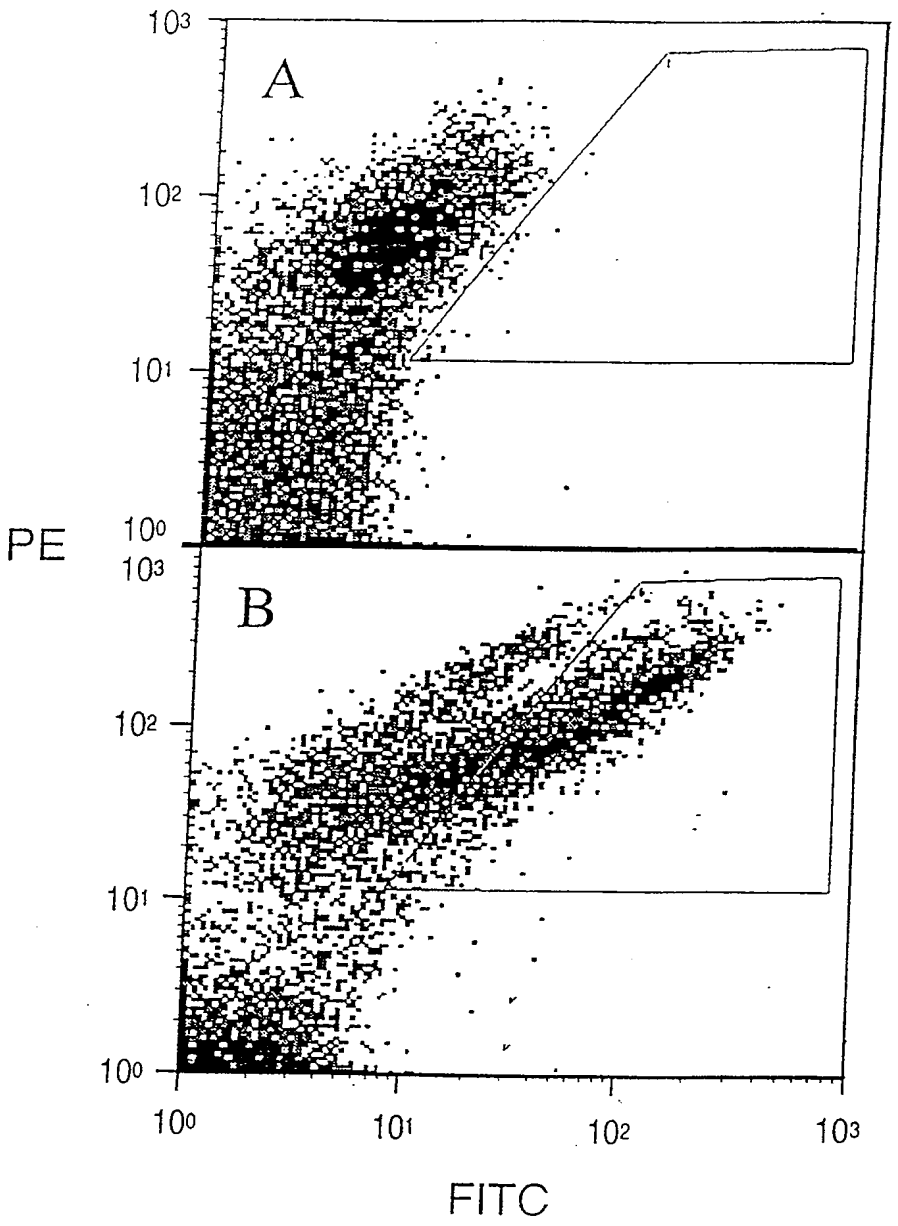


Figure 10

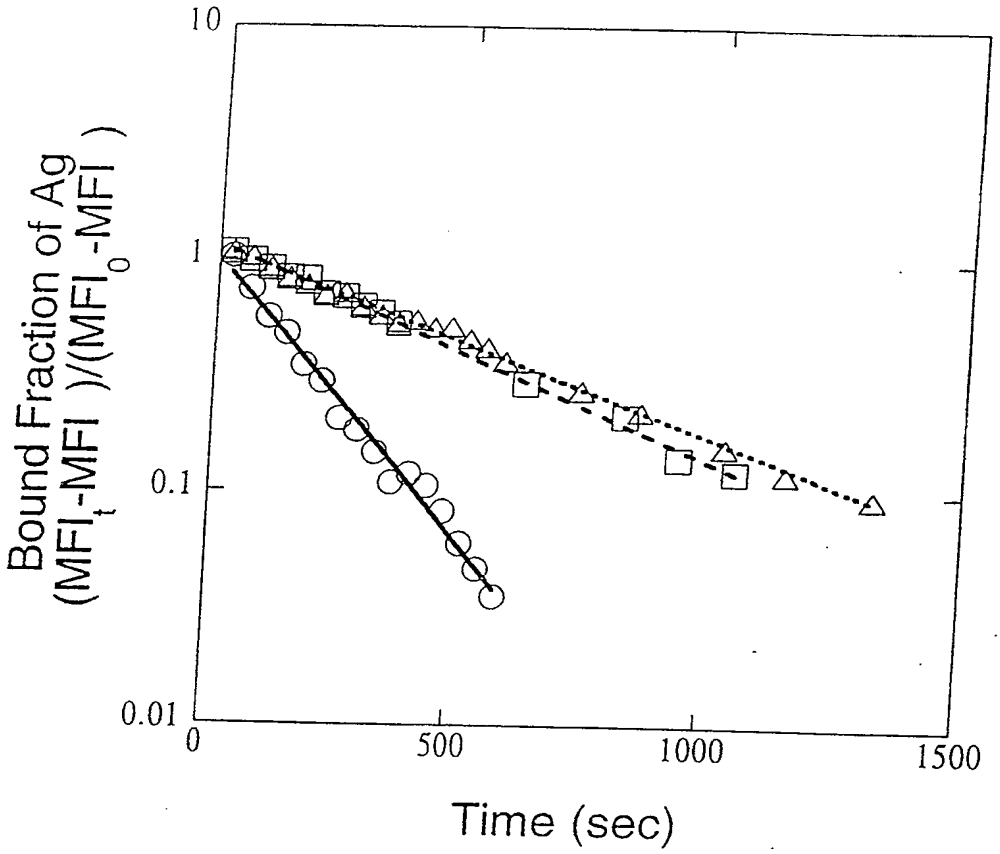

10
1
0.1
0.01
0
500
1000
1500
Time (sec)
Bound Fraction of Ag
(MFI$_t$-MFI$_\infty$)/(MFI$_0$-MFI$_\infty$)

Figure 11

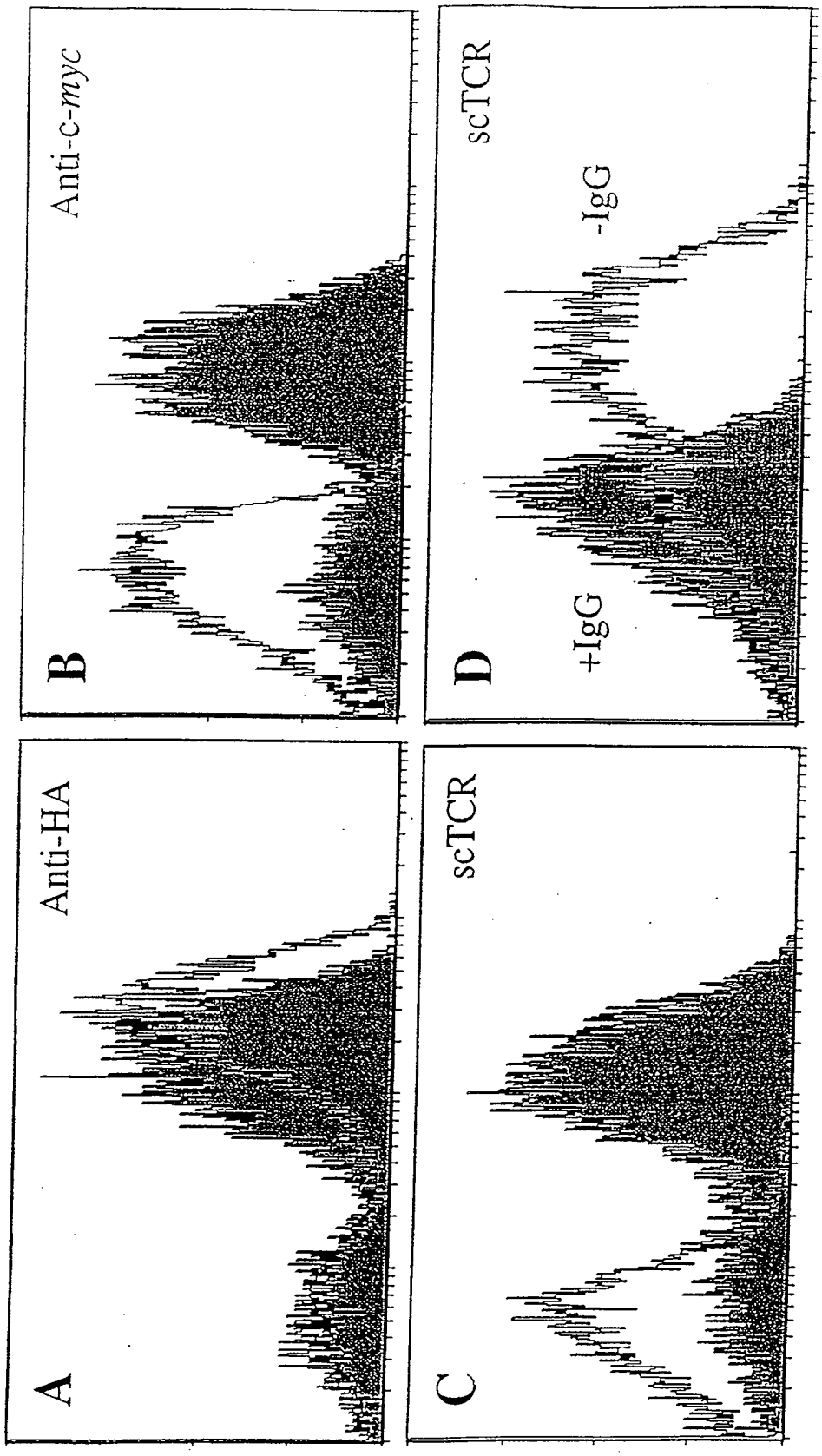


Figure 12.

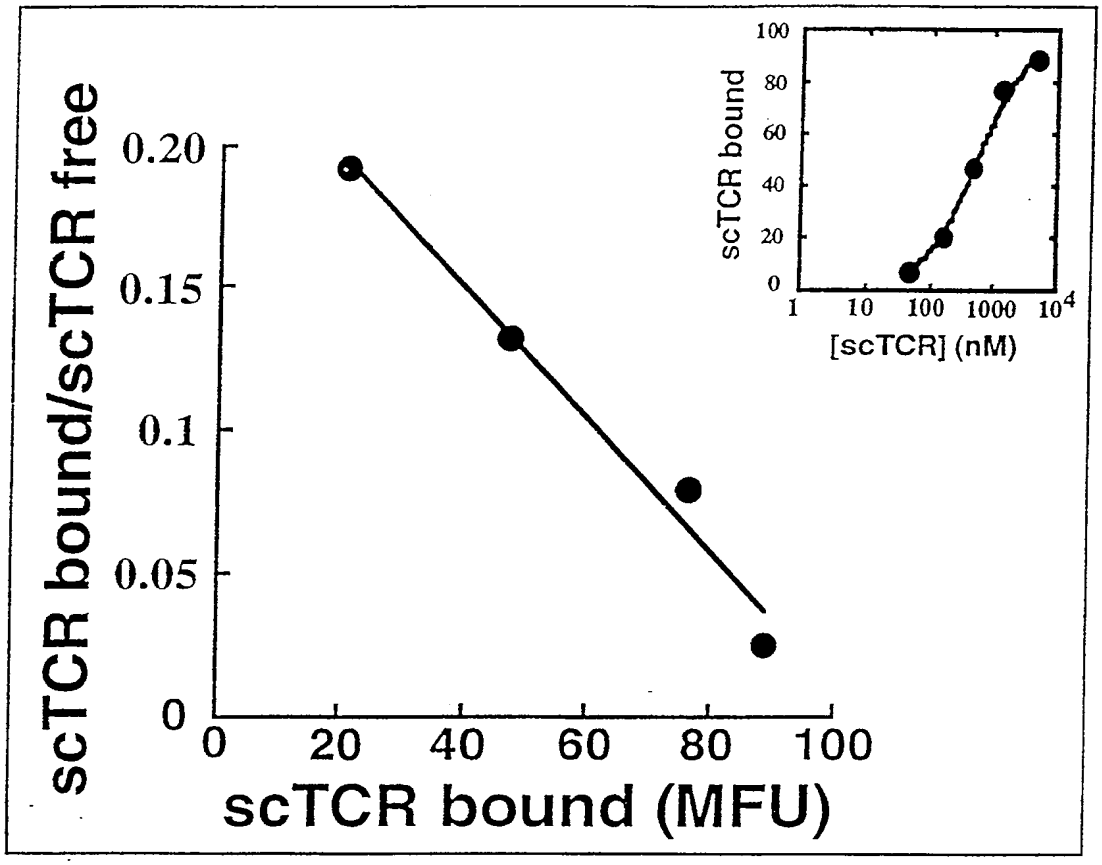

scTCR bound/scTCR free
0.20
0.15
0.1
0.05
0
0
20
40
60
80
100
scTCR bound (MFU)
scTCR bound
100
80
60
40
20
0
1
10
100
1000
10^4
[scTCR] (nM)

Figure 13

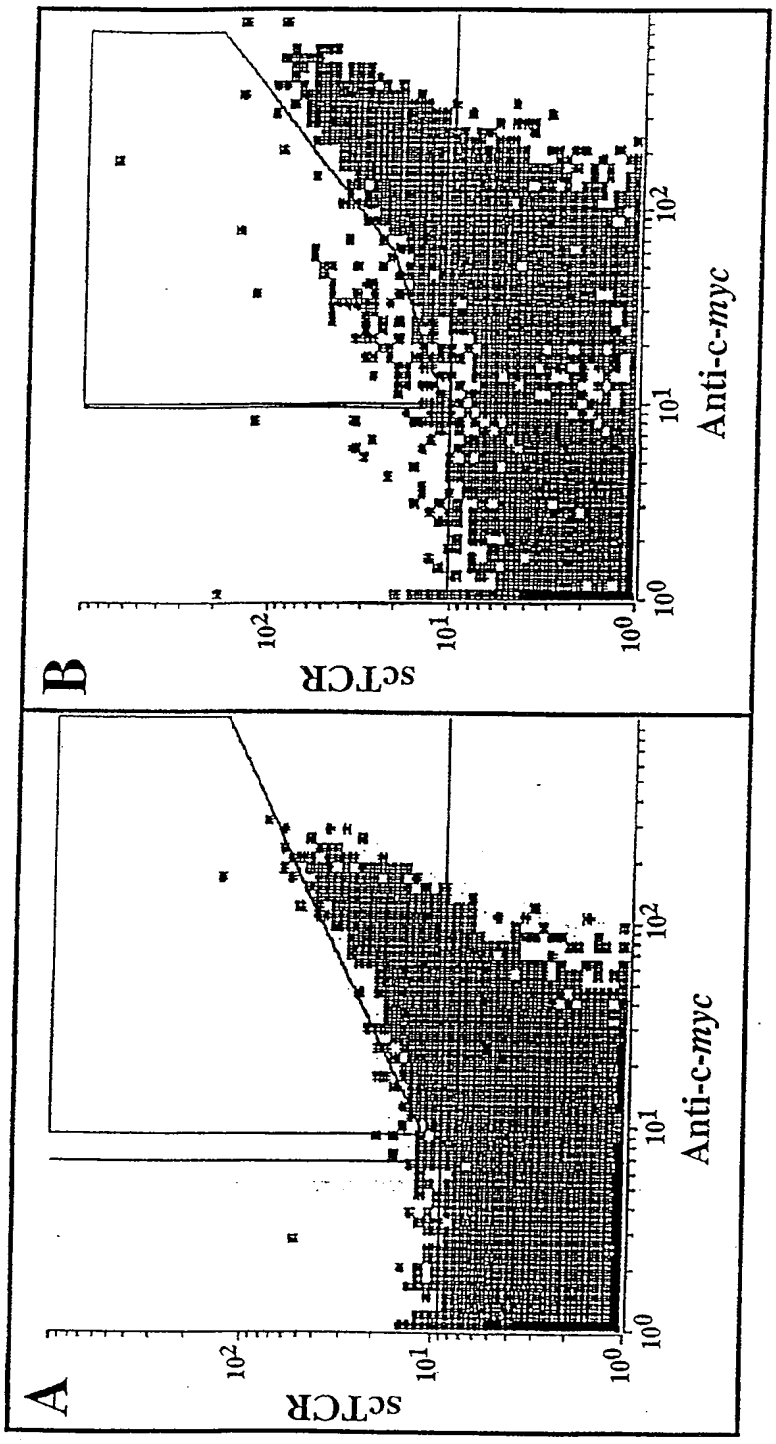


Figure 14

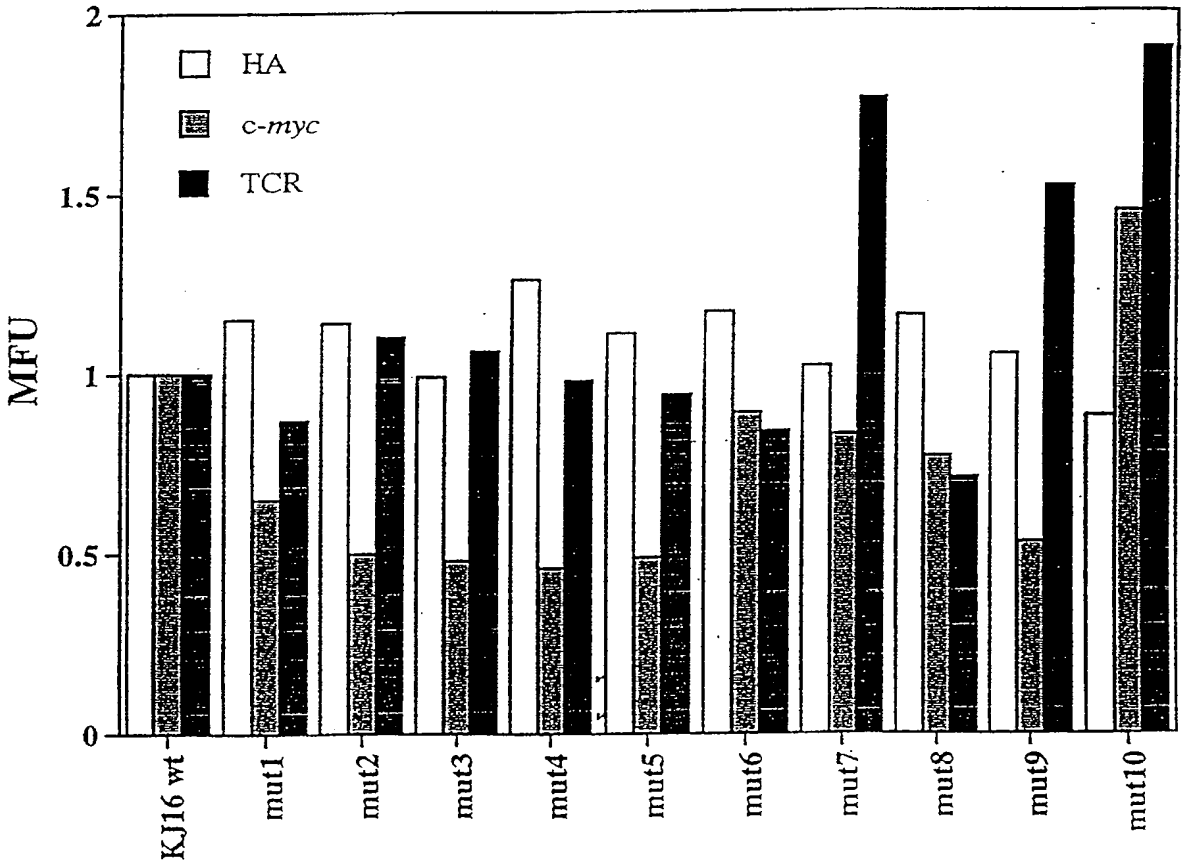

2
1.5
1
0.5
0
MFU
HA
c-myc
TCR
KJ16 wt
mut1
mut2
mut3
mut4
mut5
mut6
mut7
mut8
mut9
mut10

Figure 15

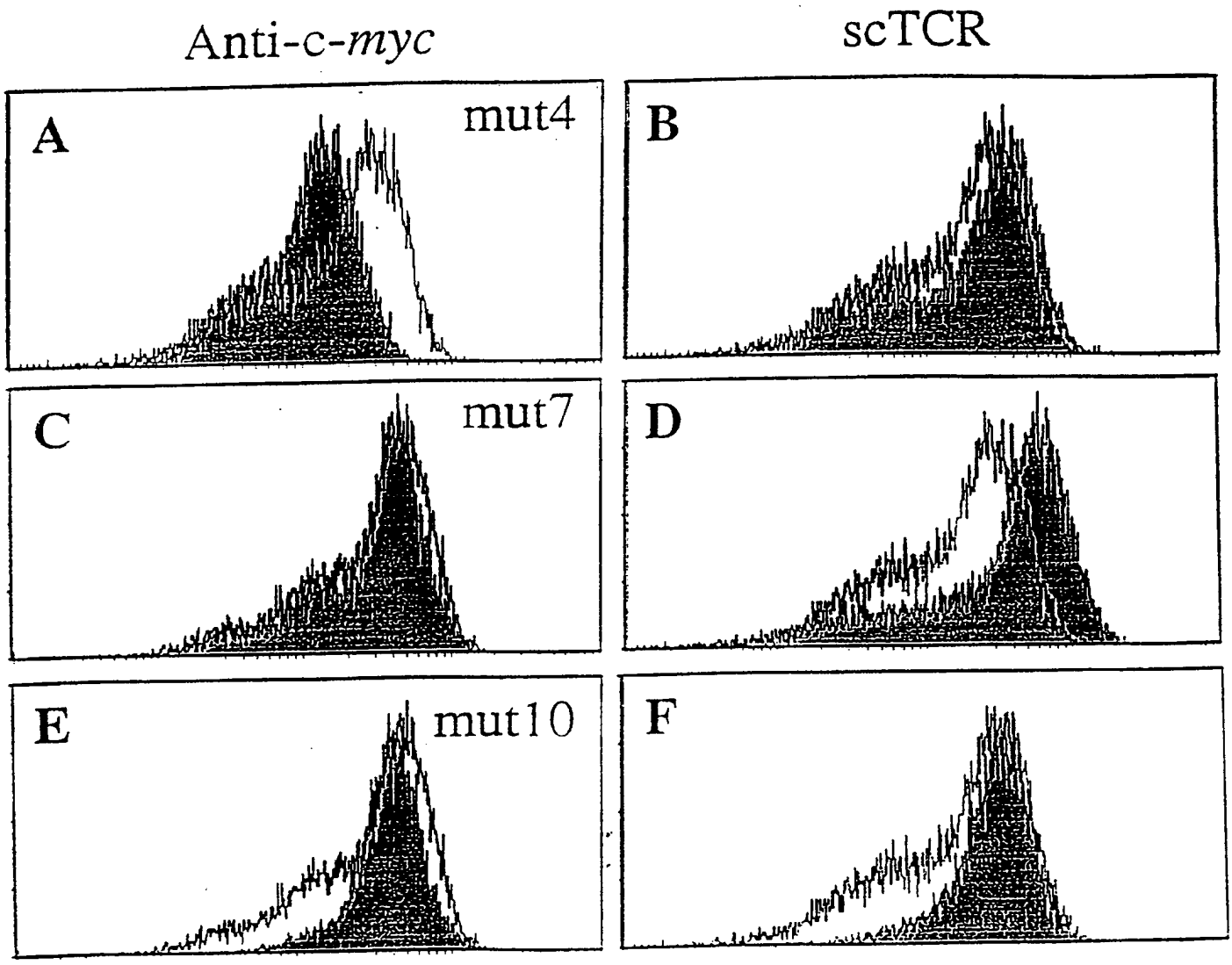


Figure 16

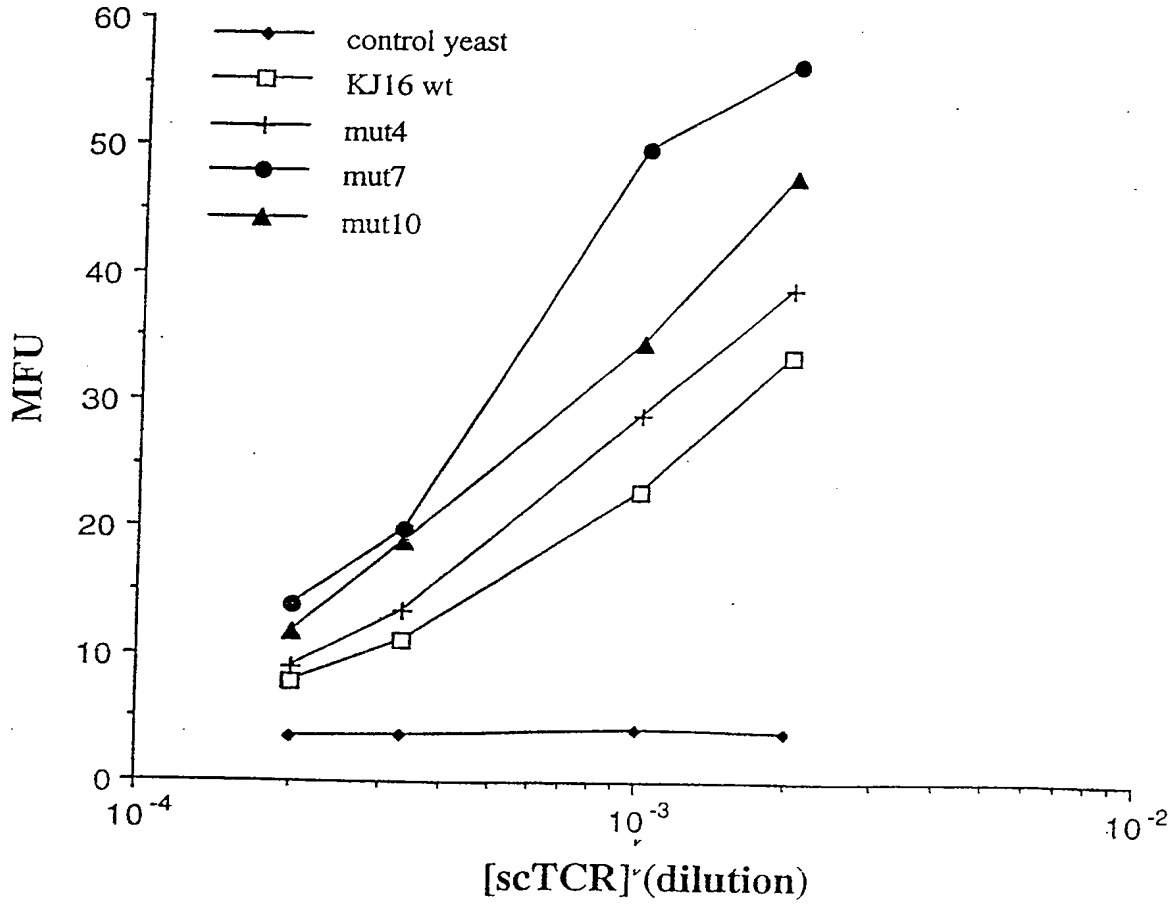

60
50
40
30
20
10
0
MFU
control yeast
KJ16 wt
mut4
mut7
mut10
10 -4
10 -3
10 -2
[scTCR] (dilution)

Figure 17

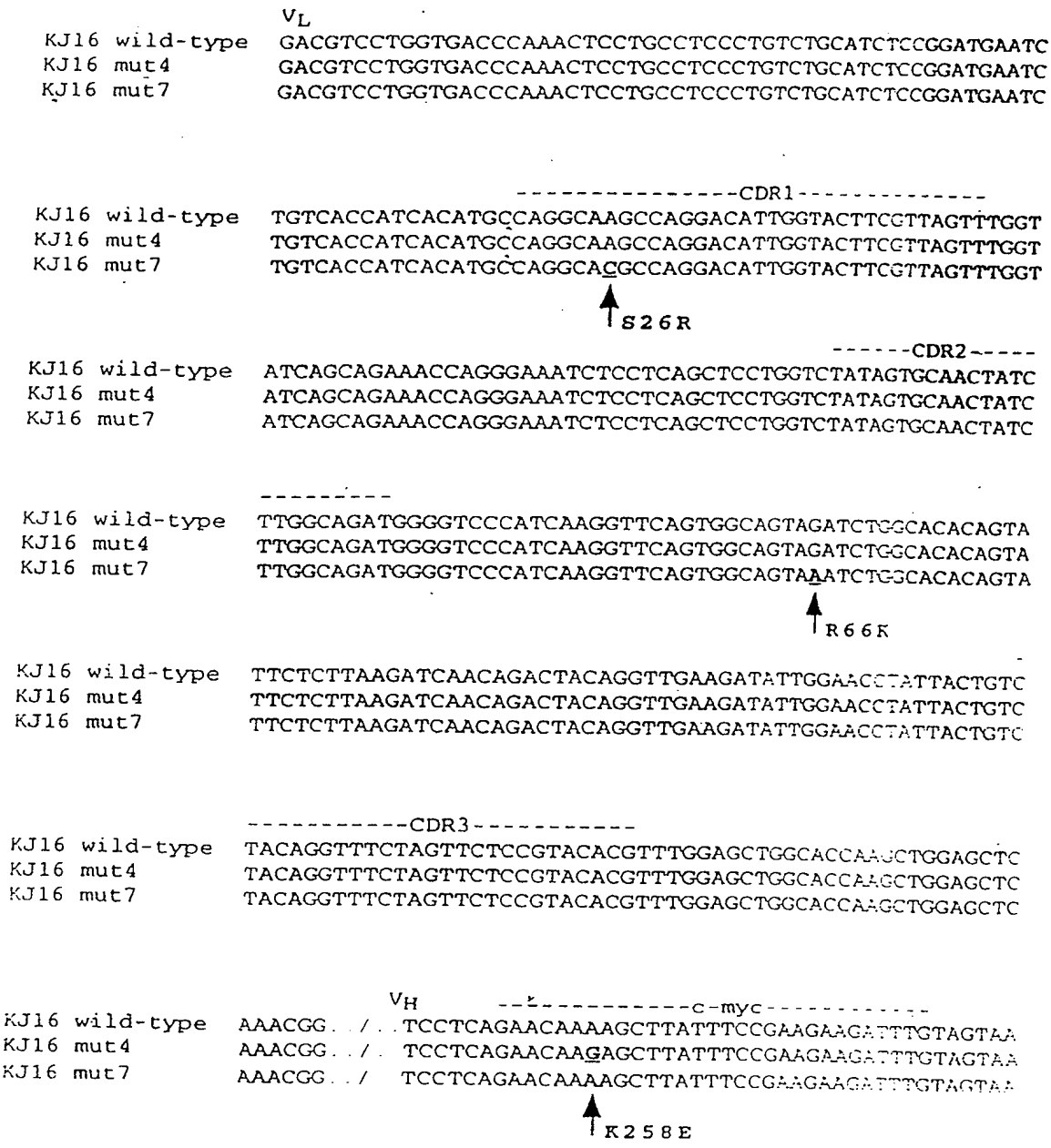

VL
KJ16 wild-type GACGTCCTGGTGACCCAAACTCCTGCCTCCCTGTCTGCATCTCCGGATGAATC
KJ16 mut4 GACGTCCTGGTGACCCAAACTCCTGCCTCCCTGTCTGCATCTCCGGATGAATC
KJ16 mut7 GACGTCCTGGTGACCCAAACTCCTGCCTCCCTGTCTGCATCTCCGGATGAATC
CDR1
KJ16 wild-type TGTCACCATCACATGCCAGGCAAGCCAGGACATTGGTACTTCGTTAGTTTGGT
KJ16 mut4 TGTCACCATCACATGCCAGGCAAGCCAGGACATTGGTACTTCGTTAGTTTGGT
KJ16 mut7 TGTCACCATCACATGCCAGGCACGCCAGGACATTGGTACTTCGTTAGTTTGGT
S26R
CDR2
KJ16 wild-type ATCAGCAGAAACCAGGGAAATCTCCTCAGCTCCTGGTCTATAGTGCAACTATC
KJ16 mut4 ATCAGCAGAAACCAGGGAAATCTCCTCAGCTCCTGGTCTATAGTGCAACTATC
KJ16 mut7 ATCAGCAGAAACCAGGGAAATCTCCTCAGCTCCTGGTCTATAGTGCAACTATC
KJ16 wild-type TTGGCAGATGGGGTCCCATCAAGGTTCAGTGGCAGTAGATCTGGCACACAGTA
KJ16 mut4 TTGGCAGATGGGGTCCCATCAAGGTTCAGTGGCAGTAGATCTGGCACACAGTA
KJ16 mut7 TTGGCAGATGGGGTCCCATCAAGGTTCAGTGGCAGTAAATCTGGCACACAGTA
R66K
KJ16 wild-type TTCTCTTAAGATCAACAGACTACAGGTTGAAGATATTGGAACCTATTACTGTC
KJ16 mut4 TTCTCTTAAGATCAACAGACTACAGGTTGAAGATATTGGAACCTATTACTGTC
KJ16 mut7 TTCTCTTAAGATCAACAGACTACAGGTTGAAGATATTGGAACCTATTACTGTC
CDR3
KJ16 wild-type TACAGGTTTCTAGTTCTCCGTACACGTTTGGAGCTGGCACCAAGCTGGAGCTC
KJ16 mut4 TACAGGTTTCTAGTTCTCCGTACACGTTTGGAGCTGGCACCAAGCTGGAGCTC
KJ16 mut7 TACAGGTTTCTAGTTCTCCGTACACGTTTGGAGCTGGCACCAAGCTGGAGCTC
VH
c-myc
KJ16 wild-type AAACGG../..TCCTCAGAACAAAAGCTTATTTCCGAAGAAGATTTGTAGTAA
KJ16 mut4 AAACGG../..TCCTCAGAACAAGAGCTTATTTCCGAAGAAGATTTGTAGTAA
KJ16 mut7 AAACGG../..TCCTCAGAACAAAAGCTTATTTCCGAAGAAGATTTGTAGTAA
K258E

Figure 1B.

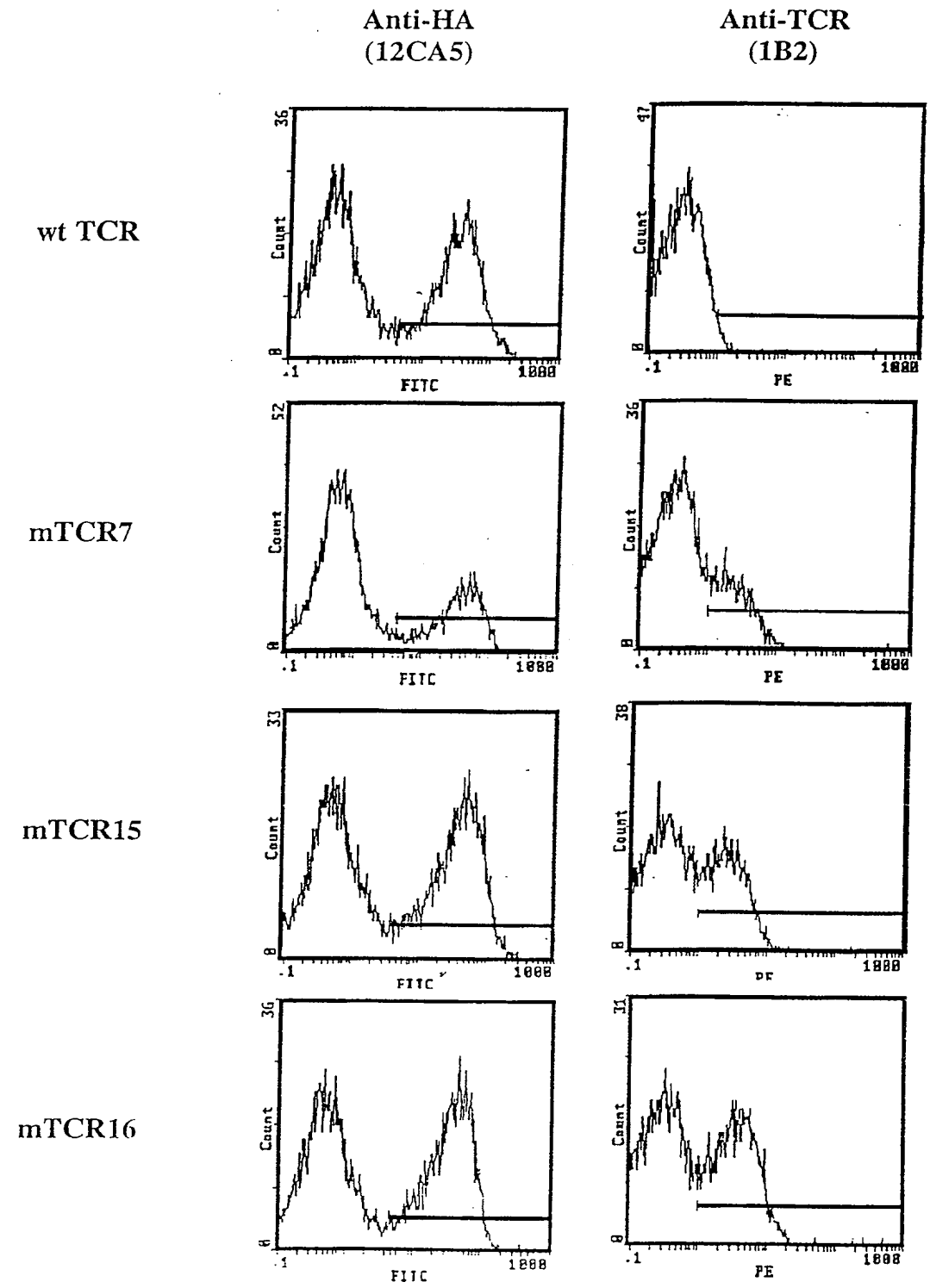

Anti-HA
(12CA5)
Anti-TCR
(1B2)
wt TCR
mTCR7
mTCR15
mTCR16
Count
FITC
PE
.1
1000

Figure 19

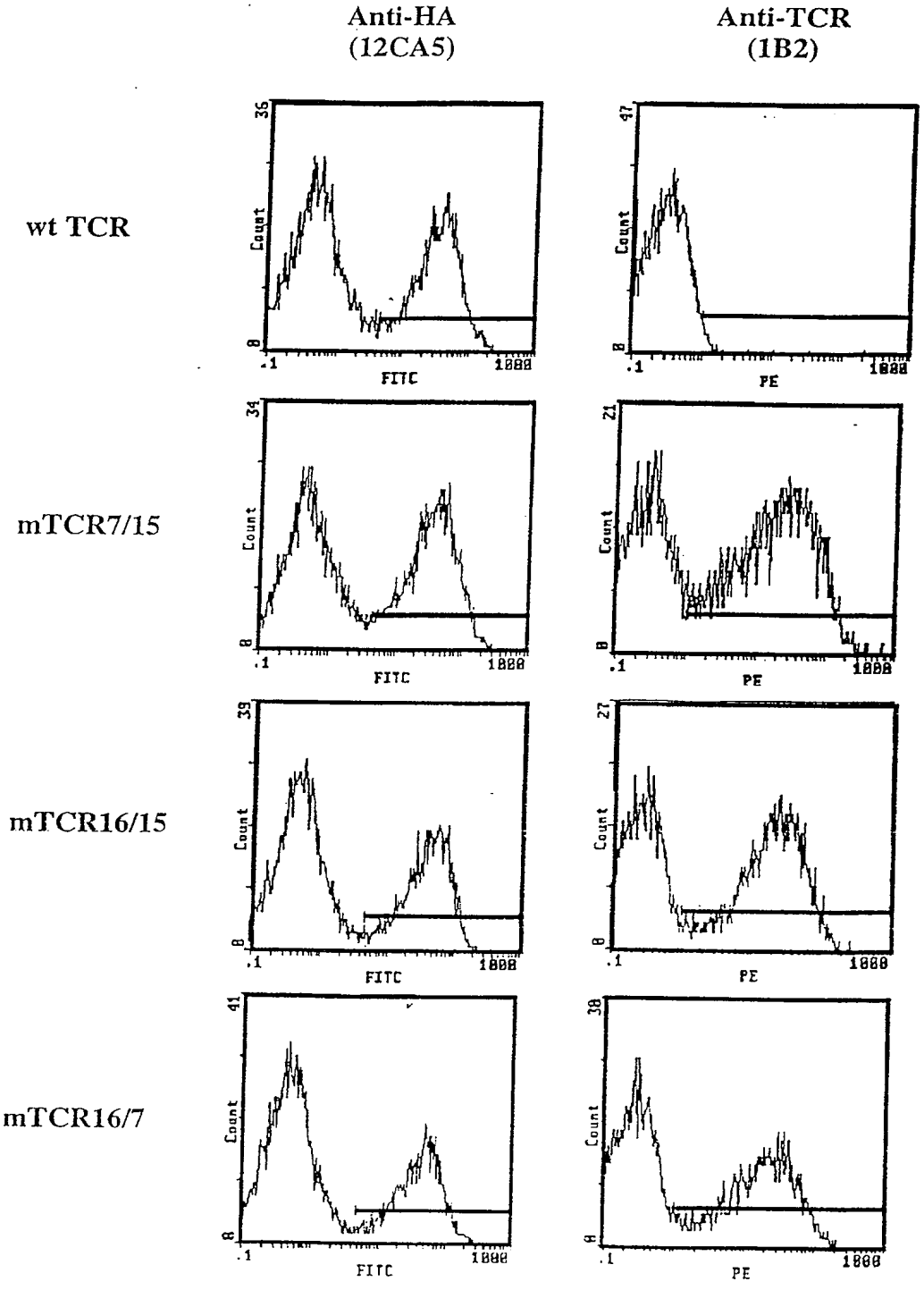

Anti-HA
(12CA5)
Anti-TCR
(1B2)
wt TCR
mTCR7/15
mTCR16/15
mTCR16/7
Count
FITC
PE
.1
1000

Figure 20

Vβ8.2
GACGTCGCAG TCACCCAAAG CCCAAGAAAC AAGGTGGCAG TAACAGGAGG AAAGGTGACA
Gly17ΔGlu
-------cdr1---------
TTGAGCTGTA ATCAGACTAA TAACCACAAC AACATGTACT GGTATCGGCA GGACACGGGG

------------cdr2-------------
CATGGGCTGA GGCTGATCCA TTATTCATAT GGTGCTGGCA GCACTGAGAA AGGAGATATC

----------hv4----------
CCTGATGGAT ACAAGGCCTC CAGACCAAGC CAAGAGAACT TCTCCCTCAT TCTGGAGTTG

----------cdr3---------
GCTACCCCCT CTCAGACATC AGTGTACTTC TGTGCCAGCG GTGGGGGGGG CACCTTGTAC

linker
TTTGGTGCGG GCACCCGACT ATCGGTGCTA TCCTCCGCGG ATGATGCTAA GAAGGATGCT

Vα3.1
GCTAAGAAGG ATGATGCTAA GAAAGATGAT GCTAAGAAAG ATGCACAGTC AGTGACACAG

CCCGATGCTC GCGTCACTGT CTCTGAAGGA GCCTCTCTGC AGCTGAGATG CAAGTATTCC

-------cdr1--------
TACTCTGCGA CACCTTATCT GTTCTGGTAT GTCCAGTACC CGCGGCAGGG GCTGCAGCTG
Leu43ΔPro
---------cdr2----------
CTCCTCAAGT ACTATTCCGG AGACCCAGTG GTTCAAGGAG TGAATGGCTT TGAGGCTGAG

--------hv4---------
TTCAGCAAGA GCAACTCTTC CTTCCACCTG CGGAAAGCCT CCGTGCACTG GAGCGACTCG

-------cdr3------
GCTGTGTACT TCTGTGCTGT GAGCGGCTTT GCAAGTGCGC TGACATTTGG ATCTGGCACA
Leu104ΔPro

AAAGTCATTG TTCTACCATA CATCTAG + 6-His

Figure 21

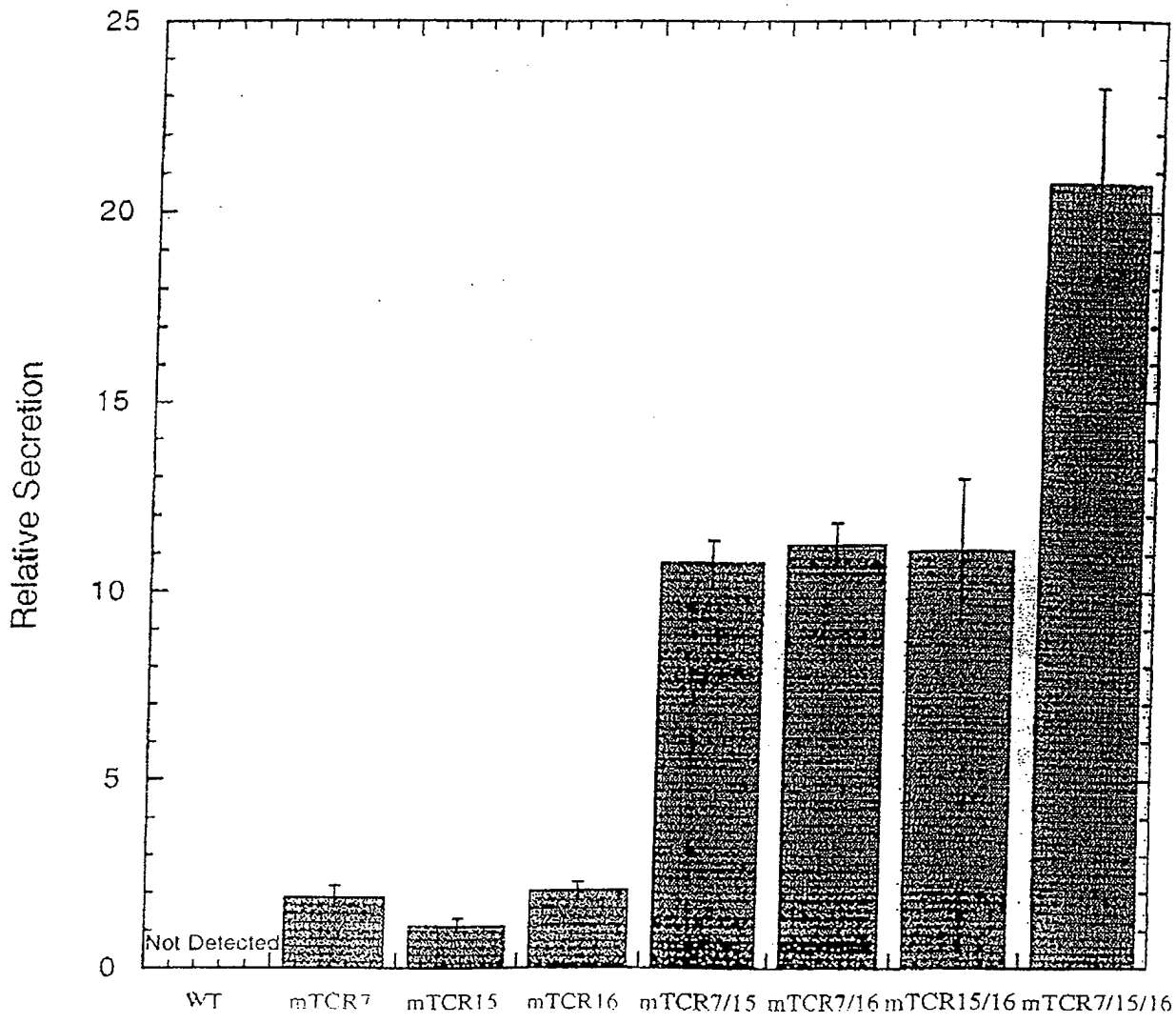

25
20
15
10
5
0
Relative Secretion
Not Detected
WT
mTCR7
mTCR15
mTCR16
mTCR7/15
mTCR7/16
mTCR15/16
mTCR7/15/16

FIGURE 22

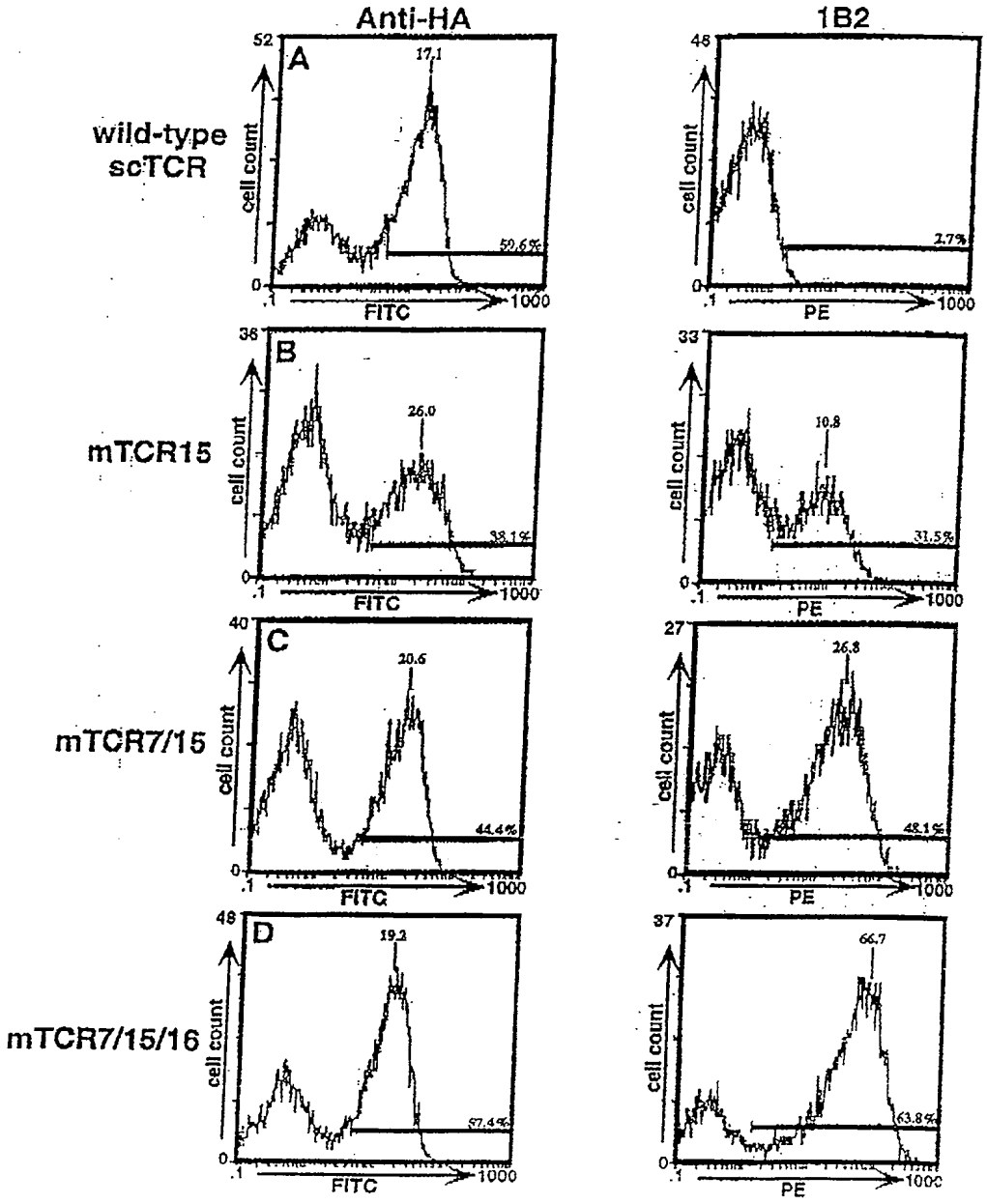

Anti-HA
1B2
wild-type scTCR
mTCR15
mTCR7/15
mTCR7/15/16
A
B
C
D
cell count
FITC
PE
17.1
59.6%
2.7%
26.0
38.1%
10.8
31.5%
20.6
44.4%
26.8
48.1%
19.2
67.4%
66.7
63.8%

FIGURE 23A

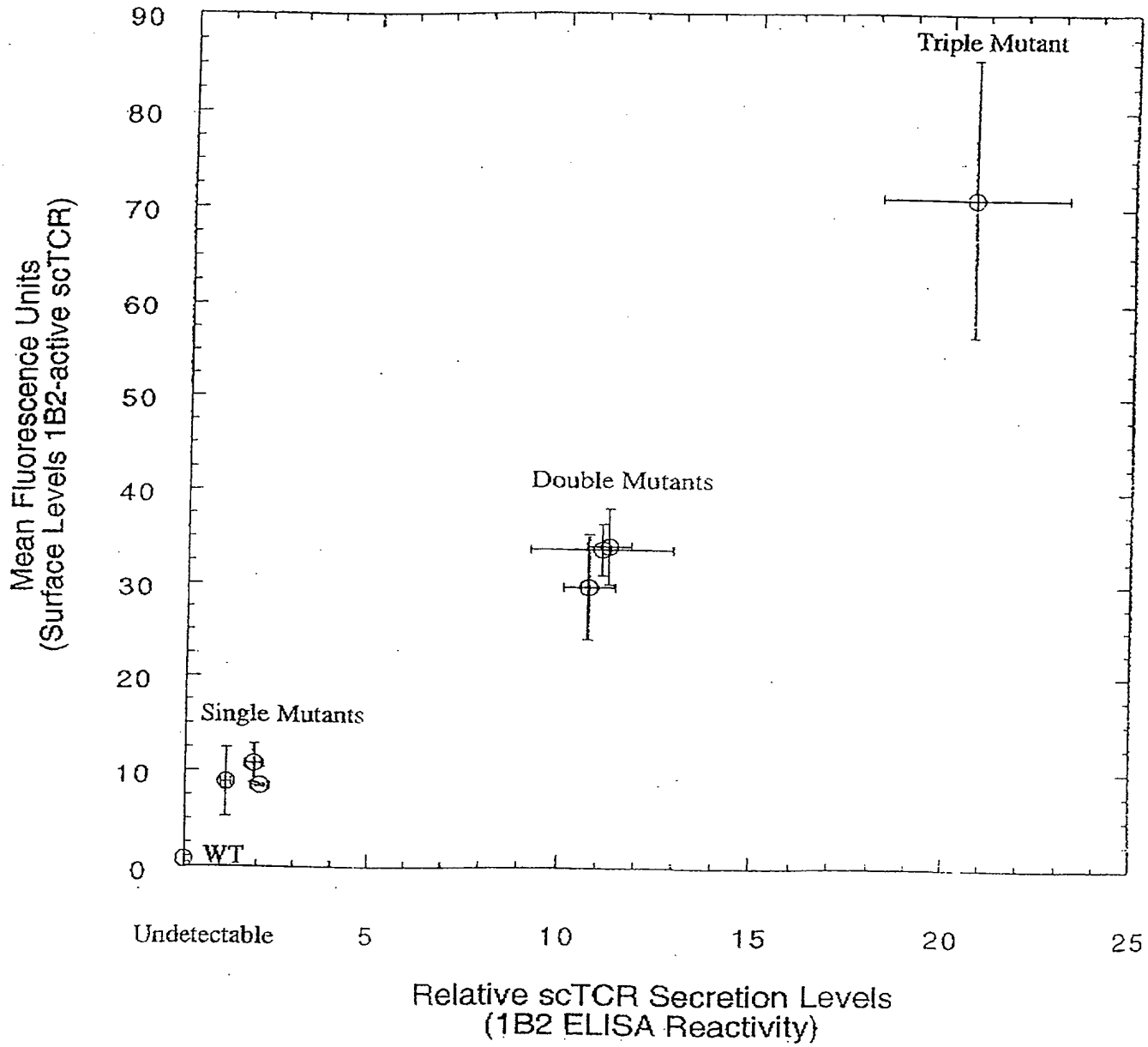

Mean Fluorescence Units
(Surface Levels 1B2-active scTCR)
90
80
70
60
50
40
30
20
10
0
Triple Mutant
Double Mutants
Single Mutants
WT
Undetectable
5
10
15
20
25
Relative scTCR Secretion Levels
(1B2 ELISA Reactivity)

FIGURE 23B

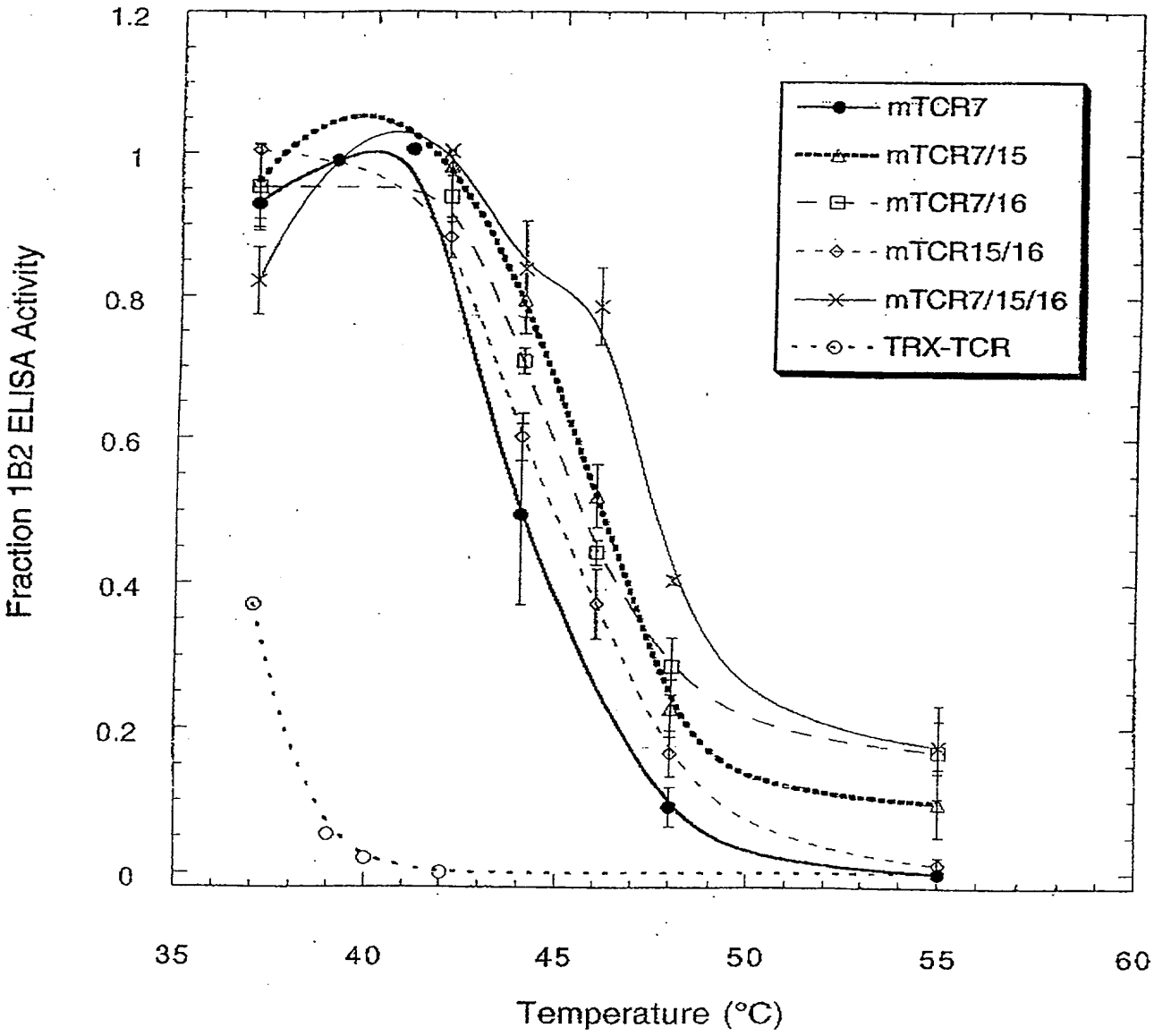

1.2
1
0.8
0.6
0.4
0.2
0
Fraction 1B2 ELISA Activity
35
40
45
50
55
60
Temperature (°C)
mTCR7
mTCR7/15
mTCR7/16
mTCR15/16
mTCR7/15/16
TRX-TCR

FIGURE 24A

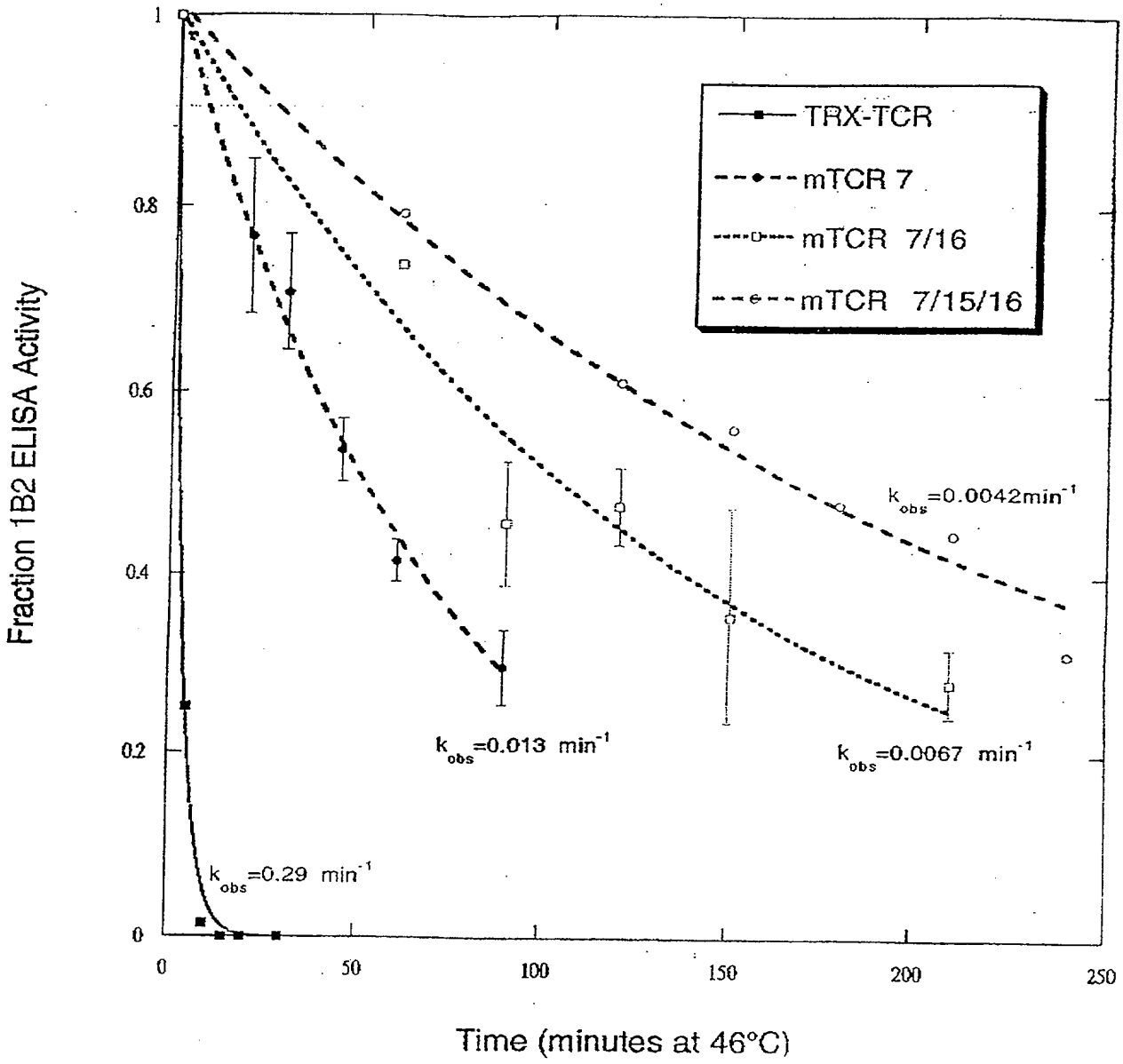

TRX-TCR
mTCR 7
mTCR 7/16
mTCR 7/15/16
Fraction 1B2 ELISA Activity
Time (minutes at 46°C)
k obs=0.0042min-1
k obs=0.013 min-1
k obs=0.0067 min-1
k obs=0.29 min-1
0
0.2
0.4
0.6
0.8
1
0
50
100
150
200
250

FIGURE 24B

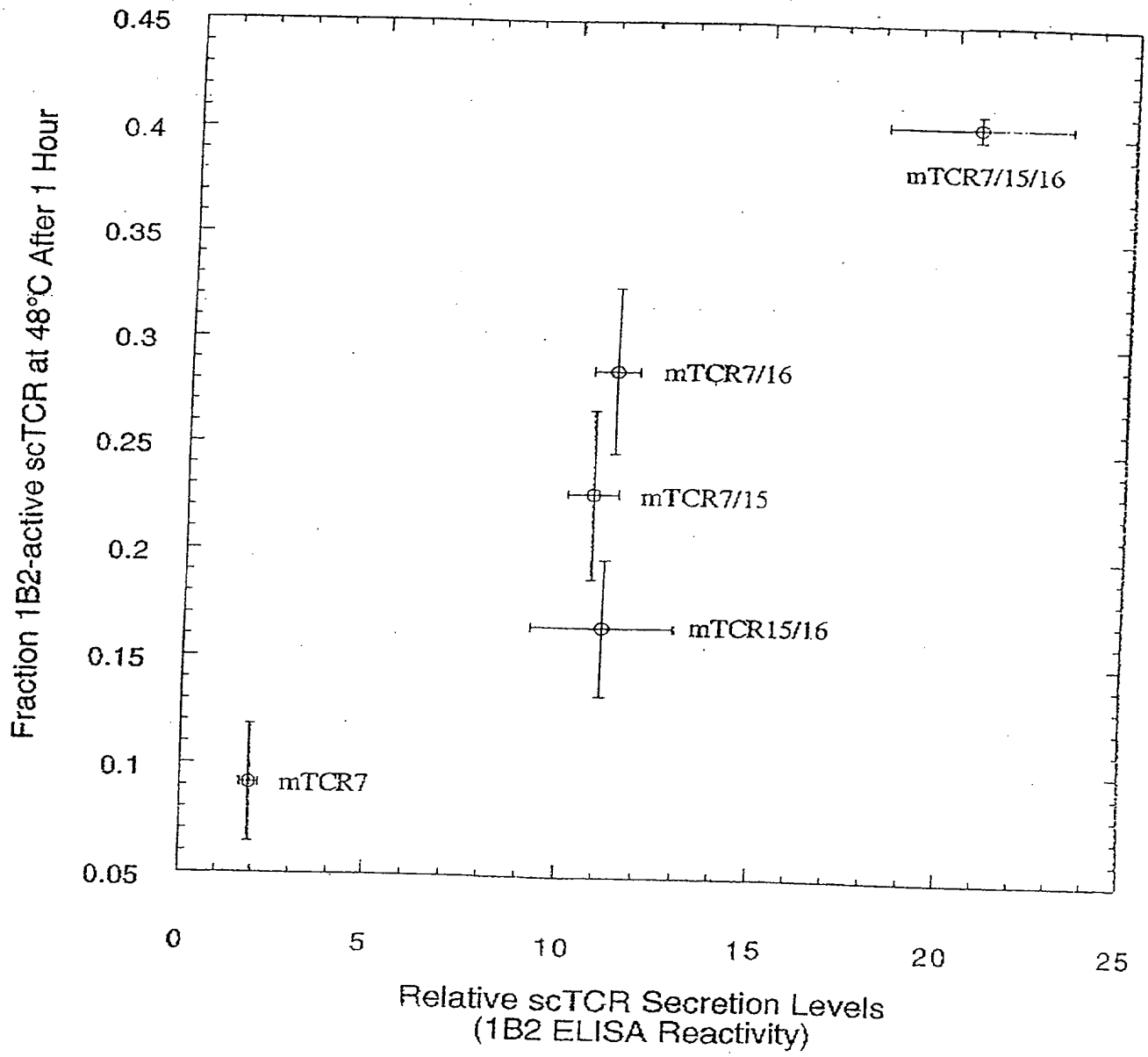

0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
Fraction 1B2-active scTCR at 48°C After 1 Hour
mTCR7/15/16
mTCR7/16
mTCR7/15
mTCR15/16
mTCR7
0
5
10
15
20
25
Relative scTCR Secretion Levels
(1B2 ELISA Reactivity)

FIGURE 25

SEQUENCE LISTING

<110> Wittrup et al.
<120> Yeast Cell Surface Display of Proteins and Uses Thereof
<130> D6061PCT
<141> 1999-01-20
<150> 09/009,388
<151> 1998-01-20
<160> 26
<170> WORD 6.0.1 for Macintosh

<210> 1
<211> 9
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 1
Tyr Pro Tyr Asp Val Pro Asp Tyr Ala
5

<210> 2
<211> 10
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 2
Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
5 10

<210> 3
<211> 6
<212> PRT
<213> Unknown
<220>
<223> Epitope tag

<400> 3
Asp Thr Tyr Arg Tyr Ile
5

<210> 4
<211> 6
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 4
Thr Asp Phe Tyr Leu Lys
5

<210> 5
<211> 9
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 5
Glu Glu Glu Glu Tyr Met Pro Met Glu
5

<210> 6
<211> 11
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 6
Lys Pro Pro Thr Pro Pro Pro Glu Pro Glu Thr
5 10

<210> 7
<211> 6
<212> PRT
<213> Unknown
<220>

<223> Epitope tag
<400> 7
His His His His His His
5

<210> 8
<211> 5
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 8
Arg Tyr Ile Arg Ser
5

<210> 9
<211> 8
<212> PRT
<213> Unknown
<220>
<223> Epitope tag
<400> 9
Asp Tyr Lys Asp Asp Asp Asp Lys
5

<210> 10
<211> 30
<212> DNA
<213> Artificial sequence
<220>
<223> PCR primer towards AGA2 gene of *S. cerevisiae*
<400> 10
attagaattc cctacttcat acattttcaa 30

<210> 11
<211> 73
<212> DNA
<213> Artificial sequence
<220>

<223> PCR primer towards AGA2 gene of *S. cerevisiae*

<400> 11

attactcgag ctattactgc agagcgtagt ctggaacgtc gtatgggtaa aaaacatact 60
gtgtgtttat ggg 73

<210> 12

<211> 71

<212> DNA

<213> Artificial sequence

<220>

<223> PCR primer towards the factor Xa recognition sequence

<400> 12

tcgacgattg aaggtagata cccatacgac gttccagact acgctctgca gtaatagatt 60
atcctcgagc t 71

<210> 13

<211> 63

<212> DNA

<213> Artificial sequence

<220>

<223> PCR primer towards the factor Xa recognition sequence

<400> 13

cgaggataat ctattactgc agagcgtagt ctggaacgtc gtatgggtat ctaccttcaa 60
tcg 63

<210> 14

<211> 10

<212> DNA

<213> Artificial sequence

<220>

<223> PCR primer towards Gal promoter

<400> 14

aattggtacc 10

<210> 15

<211> 10

<212> DNA

<213> Artificial sequence
<220>
<223> PCR primer towards Gal promoter
<400> 15
gatcgaattc 10

<210> 16
<211> 34
<212> DNA
<213> Artificial sequence
<220>
<223> PCR primer towards 4-4-20 scFv
<400> 16
ggttggccaa gctagcgacg tcgttatgac tcaa 34

<210> 17
<211> 70
<212> DNA
<213> Artificial sequence
<220>
<223> PCR primer towards 4-4-20 scFv
<400> 17
ggccggccaa ctcgagctat tacaagtctt cttcagaaat aagcttttgt tctgaggaga 60
cggtgactga 70

<210> 18
<211> 30
<212> DNA
<213> Artificial sequence
<220>
<223> PCR primer towards AGA1 gene of *S. cerevisiae*
<400> 18
attagaattc agctaaaaaa accaaaaaat 30

<210> 19
<211> 33
<212> DNA
<213> Artificial sequence
<220>

<223> PCR primer towards AGA1 gene of *S. cerevisiae*
<400> 19
attactcgag ctattaactg aaaattacat tgc 33

<210> 20
<211> 15
<212> PRT
<213> Artificial sequence
<220>
<223> Linker between PCR products to maintain correct reading frame
<400> 20
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
5 10 15

<210> 21
<211> 1172
<212> DNA
<213> Artificial sequence
<220>
<223> Cloned PCR products to produce AFA2-HA-4-4-20 gene cassette
<400> 21
attagaattc cctacttcat acattttcaa ttaagatgca gttacttcgc tgtttttcaa 60
tattttctgt tattgcttca gttttagcac aggaactgac aactatatgc gagcaaatcc 120
cctcaccaac tttagaatcg acgccgtact ctttgtcaac gactactatt ttggccaacg 180
ggaaggcaat gcaaggagtt tttgaatatt acaaatcagt aacgtttgtc agtaattgcg 240
gttctcaccc ctcaacaact agcaaaggca gccccataaa cacacagtat gtttttaagg 300
acaatagctc gacgattgaa ggtagatacc catacgacgt tccagactac gctctgcagg 360
ctagcgacgt cgttatgact caaacaccac tatcacttcc tgttagtcta ggagatcaag 420
cctccatctc ttgcagatct agtcagagcc ttgtacacag taatggaaac acctatttac 480
gttggtacct gcagaagcca ggccagtctc caaaggtcct gatctacaaa gtttccaacc 540
gattttctgg ggtcccagac aggttcagtg gcagtggatc agggacagat ttcacactca 600
agatcagcag agtggaggct gaggatctgg gagtttattt ctgctctcaa agtacacatg 660
ttccgtggac gttcggtgga ggcaccaagc ttgaaattaa gtcctctgct gatgatgcta 720
agaaggatgc tgctaagaag gatgatgcta agaaagatga tgctaagaaa gatggtgacg 780
tcaaactgga tgagactgga ggaggcttgg tgcaacctgg gaggcccatg aaactctcct 840
gtgttgcctc tggattcact tttagtgact actggatgaa ctgggtccgc cagtctccag 900

agaaaggact ggagtgggta gcacaaatta gaaacaaacc ttataattat gaaacatatt 960
attcagattc tgtgaaaggc agattcacca tgtcaagaga tgattccaaa agtagtgtct 1020
acctgcaaat gaacaactta agagttgaag acatgggtat ctattactgt acgggttctt 1080
actatggtat ggactactgg ggtcaaggaa cctcagtcac cgtctcctca gaacaaaagc 1140
ttatttctga agaagacttg taatagctcg ag 1172

<210> 22
<211> 366
<212> DNA
<213> Artificial sequence
<220>
<223> PCR products of wild type scFv-KJ16
<400> 22
gacgtcctgg tgacccaaac tcctgcctcc ctgtctgcat ctccggatga atctgtcacc 60
atcacatgcc aggcaagcca ggacattggt acttcgttag tttggtatca gcagaaacca 120
gggaaatctc ctcagctcct ggtctatagt gcaactatct tggcagatgg ggtcccatca 180
aggttcagtg gcagtagatc tggcacacag tattctctta agatcaacag actacaggtt 240
gaagatattg gaacctatta ctgtctacag gtttctagtt ctccgtacac gtttggagct 300
ggcaccaagc tggagctcaa acggtcctca gaacaaaagc ttatttccga agaagatttg 360
tagtaa 366

<210> 23
<211> 366
<212> DNA
<213> Artificial sequence
<220>
<223> PCR products of KJ16-mut4
<400> 23
gacgtcctgg tgacccaaac tcctgcctcc ctgtctgcat ctccggatga atctgtcacc 60
atcacatgcc aggcaagcca ggacattggt acttcgttag tttggtatca gcagaaacca 120
gggaaatctc ctcagctcct ggtctatagt gcaactatct tggcagatgg ggtcccatca 180
aggttcagtg gcagtagatc tggcacacag tattctctta agatcaacag actacaggtt 240
gaagatattg gaacctatta ctgtctacag gtttctagtt ctccgtacac gtttggagct 300
ggcaccaagc tggagctcaa acggtcctca gaacaagagc ttatttccga agaagatttg 360
tagtaa 366

<210> 24
<211> 366
<212> DNA

<213> Artificial sequence
<220>
<223> PCR products of KJ16-mut7
<400> 24

gacgtcctgg tgacccaaac tcctgcctcc ctgtctgcat ctccggatga atctgtcacc 60
atcacatgcc aggcacgcca ggacattggt acttcgttag tttggtatca gcagaaacca 120
gggaaatctc ctcagctcct ggtctatagt gcaactatct tggcagatgg ggtcccatca 180
aggttcagtg gcagtaaatc tggcacacag tattctctta agatcaacag actacaggtt 240
gaagatattg gaacctatta ctgtctacag gtttctagtt ctccgtacac gtttggagct 300
ggcaccaagc tggagctcaa acggtcctca gaacaaaagc ttatttccga agaagatttg 360
tagtaa 366

<210> 25
<211> 747
<212> DNA
<213> Artificial sequence
<220>
<223> Sequence showing mutations in T-cell receptor
<400> 25

gacgtcgcag tcacccaaag cccaagaaac aaggtggcag taacaggagg aaaggtgaca 60
ttgagctgta atcagactaa taaccacaac aacatgtact ggtatcggca ggacacgggg 120
catgggctga ggctgatcca ttattcatat ggtgctggca gcactgagaa aggagatatc 180
cctgatggat acaaggcctc cagaccaagc caagagaact tctccctcat tctggagttg 240
gctacccccc ctcagacatc agtgtacttc tgtgccagcg gtgggggggg caccttgtac 300
tttggtgcgg gcacccgact atcggtgcta tcctccgcgg atgatgctaa gaaggatgct 360
gctaagaagg atgatgctaa gaaagatcat gctaagaaag atgcacagtc agtgacacag 420
cccgatgctc gcgtcactgt ctctgaagga gcctctctgc agctgagatg caagtattcc 480
tactctgcga caccttatct gttctggtat gtccagtacc cgcggcaggg gctgcagctg 540
ctcctcaagt actattccgg agacccagtg gttcaaggag tgaatggctt tgaggctgag 600
ttcagcaaga gcaactcttc cttccacctg cggaaagcct ccgtgcactg gagcgactcg 660
gctgtgtact tctgtgctgt gagcggcttt gcaagtgcgc tgacatttgg atctggcaca 720
aaagtcattg ttctaccata catctag 747

<210> 26
<211> 4
<212> PRT
<213> Unknown
<220>
<223> Epitope tag

<400> 26
Ile Glu Gly Arg